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CONTRACT NUMBER: DAMD17-93-C-3004

TITLE: Preclinical Pharmacodynamic and Pharmacokinetic Studies of Investigational New Drugs

PRINCIPAL INVESTIGATOR: Patricia E. Noker, Ph.D.

CONTRACTING ORGANIZATION: Southern Research Institute
Birmingham, AL 35255-5305

REPORT DATE: November 1995

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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19960508 066

REPORT DOCUMENTATION PAGE	Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE November 1995	3. REPORT TYPE AND DATES COVERED Annual 2 Nov 94 – 1 Nov 95

4. TITLE AND SUBTITLE

Preclinical Pharmacodynamic and Pharmacokinetic Studies of Investigational New Drugs

5. FUNDING NUMBERS

DAMD17-93-C-3004

6. AUTHOR(S)

Patricia E. Noker, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Southern Research Institute
Birmingham, Alabama 35255-5305

8. PERFORMING ORGANIZATION REPORT NUMBER

SRI-LIF-95-830-7869

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

During the past year of this contract, efforts were focused on isolating and identifying the urinary and fecal metabolites eliminated following administration of either [^{14}C]HI-6, [^{14}C]PAHP or [^{14}C]WR242511 to dogs and to rats (PAHP only). Identification of the metabolites was based on mass spectral, UV absorbance and chromatographic data. Metabolites eliminated by dogs following administration of [^{14}C]HI-6 have been tentatively identified and include 2-pyridine aldoxime, a pyridone derivative of HI-6 and picolinic acid. In dogs administered [^{14}C]PAHP, evidence was obtained for the elimination 6-[4'-hydroxyaminobenzoyl]hexanol and 6-[4'-hydroxyaminobenzoyl]hexanoate, as well as other radiolabeled metabolites, in urine. In rats administered [^{14}C]PAHP, one urinary metabolite was tentatively identified as 6-[4'-hydroxyaminobenzoyl]hexanol. Preliminary data indicate that following administration of [^{14}C]WR242511 to dogs, at least one metabolite eliminated in urine is a dealkylated derivative of the compound. Further efforts to isolate and identify the other urinary metabolites of these compounds, and the fecal metabolites of PAHP, are in progress.

14. SUBJECT TERMS

Metabolite identification, urine, feces, HI-6, PAHP, WR242511

15. NUMBER OF PAGES
73

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Patricia E. Mohler 11/14/95
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I. INTRODUCTION

The overall objective of this contract is to perform studies in rodents, dogs and/or monkeys on the pharmacokinetic and pharmacodynamic properties of drugs which are under development by the U.S. Army Medical Research and Development Command. Work under this contract is executed on a task order basis. The pharmacokinetic aspect of the investigations involves an assessment of the absorption, disposition, metabolism (biotransformation) and elimination of test compounds in experimental animals. The pharmacodynamic aspect involves relating certain measured parameters, for example, the production of methemoglobin, to blood and plasma levels of test compound and/or metabolites.

Information derived from these studies is intended to provide a data base for establishing an appropriate species and appropriate doses for subsequent subchronic and chronic toxicity studies, predicting possible organ toxicities which might occur and making critical decisions concerning the continued development of a drug. In addition, the studies generate data required by the Food and Drug Administration prior to submission of a Notice of Claimed Investigational Exemption for a New Drug (IND) and New Drug Applications, Human Use (NDA).

Previously, during the second year of this contract, pharmacokinetic/pharmacodynamic and metabolism studies were initiated on three compounds which have shown potential as either anti-cyanotic agents or antidotes to anticholinesterase (nerve) agents. Specifically, the agents which have been studied, and the corresponding number for the relevant task order, are as follows:

- **WR242511**: Task Order 93-02; Single dose i.v. and oral pharmacokinetic, pharmacodynamic, bioavailability and metabolism study of WR242511 (an anti-cyanotic agent) in dogs.

- **HI-6(WR249655)**: Task Order 93-03; Single dose i.m. pharmacokinetic and metabolism study of HI-6 (a nerve agent antidote/antimalarial agent) in dogs.

- **PAHP(p-aminoheptanophenone, WR269410)**: Task Orders 94-04 and 94-05; Single dose i.v. and oral pharmacokinetic, pharmacodynamic, bioavailability and metabolism studies of PAHP (an anticyanotic agent) in dogs (T.O. 94-04) and in rats (T.O. 94-05).

In each of these studies, a radiolabeled formulation of the test compound has been utilized. For each study, the basic approach has been as follows:

a.) to determine the concentration of radioactivity in blood, red blood cells and plasma at various times after administration of the radiolabeled test compound.

b.) to determine the time-concentration profile of unchanged test compound and metabolites in plasma.

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c.)   to determine the rate and extent of urinary and fecal elimination of radiolabel.

d.)   for the anticyanotic agents, to follow the time course of methemoglobin production.

e.)   to isolate and identify the major metabolites of the test compound excreted in urine and feces.

In our 1994 annual report, data was presented relevant to the blood, plasma, urine and feces distribution and elimination of these agents and to the pharmacodynamics of these agents. During the past year of this contract, the final aspect of these studies, i.e, the isolation and identification of the urinary and fecal metabolites of these agents, has been investigated. The results of these efforts will be the focus of this report.

Background information on WR242511, HI-6 and PAHP, as well information relevant to USAMRDC's interest in developing these agents for clinical use, is described below.

●**Anti-Cyanotic Agents**

Cyanide is a rapidly acting toxic compound. Cyanide has a high affinity for iron in the ferric state and, when absorbed, reacts readily with the trivalent iron of cytochrome oxidase in mitochondria resulting in the inhibition of cellular respiration[1]. Victims exposed to cyanide may die within minutes of exposure. Thus, the high reactivity of cyanide dictates that the treatment of cyanide intoxication must be rapid to be effective.

Because of its ability to form complexes with metals, cyanide has wide industrial use in metallurgy, electroplating and metal cleaning. It is also present in agricultural products such as insecticides and rodenticides. These applications of cyanide provide potential for inadvertent exposure of humans to the compound. In addition, cyanide is released during the combustion of nitrogen-containing plastics, from cyanogenic glycosides in certain plants and fruits and from medications, such as sodium nitroprusside, which is used in treating hypotension.

Agents useful in treating cyanide toxicity include sodium nitrite, 4-dimethylaminophenol, cobalt EDTA and hydroxocobalamin[1]. Sodium nitrite and 4-dimethylaminophenol oxidize hemoglobin to methemoglobin, which competes with cytochrome oxidase for the cyanide ion. Sodium thiosulfate is commonly administered in conjunction with sodium nitrite to accelerate the conversion of cyanide to thiocyanate which is nontoxic and excreted in urine. Cobalt compounds, such as cobalt EDTA, and hydroxocobalamin have a high affinity for cyanide and act directly as cyanide chelators[2].

Hydrogen cyanide is considered to be a serious chemical warfare threat[3] and, thus, antidotes to its toxicity are of interest to the U.S. Army. While all of the above treatments are effective against cyanide toxicity, they require parental administration and careful monitoring by skilled medical personnel. In military settings the administration of any of these agents would be virtually impossible because of the high number of exposed personnel, the short time span in which the antidote needs to be delivered and the physical limitations of the protective uniform. Therefore, a prophylactic agent for cyanide poisoning would be the treatment of

choice to avert mass casualties. The ideal agent would be effective when administered daily or less frequently, would have minimal side effects and would not interfere with aerobic and anaerobic work loads necessitated in battle field settings.

The 8-aminoquinoline compound, WR242511, is an analogue of the widely used anti-malarial agent, primaquine, and was initially developed as a potential replacement drug for primaquine[4]. In addition to its activity against *Plasmodia* infections, WR242511 has been shown to produce methemoglobin at levels which are protective against cyanide poisoning in animals. When given on a 48 hour dosing schedule, WR242511 maintains methemoglobin levels between 5% and 8%[5]. These levels are effective against multiple LD_{50}s of cyanide. Thus, WR242511 is a potential agent useful for the prophylaxis and treatment of cyanide toxicity.

Information has been obtained on the pharmacokinetics and pharmacodynamics of WR242511. In dogs administered either a single i.v. dose of 2.5 mg/kg of [^{14}C]WR242511 or a single oral dose of 15 mg/kg, the terminal half-life for the disappearance of radioactivity from plasma ranged from 98 to 133 hr[6]. During a study in which plasma levels of unchanged WR242511 were measured, Marino et al determined the elimination half-life of WR242511 in plasma to be about 26-28 hr following either oral or i.v. administration of 7 mg/kg of WR242511 to dogs[5]. In addition, these investigators demonstrated that peak concentrations of WR242511 occurred within 4 hr after oral administration of the drug, whereas, peak methemoglobin levels were not achieved until 72-96 hr after dosing by either the oral or intravenous route. These data suggested that a metabolite of WR242511, which was sequestered in red blood cells, was responsible for production of the observed methemoglobinemia. In a bioavailability study conducted in our laboratory[7] in which six dogs were administered a single oral or i.v. dose of 7 mg/kg of [^{14}C]WR242511, peak blood levels of radioactivity were achieved at 76 hr after dosing by either route. In contrast, peak plasma levels of WR242511 occurred between 6 to 12 hr after oral dosing and immediately after completion of i.v. dosing. Maximum methemoglobin concentrations in blood were observed at the time of peak blood levels of radioactivity. At this time, most of the radioactivity in blood was found to be associated with the red blood cells. These results further indicated that a metabolite of WR242511 which is sequestered in red blood cells is responsible for the methemoglobinemia produced by the compound. Following either oral or i.v. administration of [^{14}C]WR242511, radioactivity was eliminated from blood in two apparent phases with mean half-lives in the range of 65 hr to 70 hr during the first phase of elimination and 385 hr to 415 hr during the terminal phase of elimination; in the same animals, a single terminal phase of elimination, with a mean half-life of 23.5 hr, was observed for unchanged WR242511. The mean oral bioavailability of radioactivity derived from [^{14}C]WR242511 was estimated to be 91.1%. The mean oral bioavailability of WR242511 was estimated to be 94.8%. Urinary and fecal excretion data demonstrated that, after either oral or i.v. administration of [^{14}C]WR242511, from 30% to 37% of the dose was excreted into urine and from 47% to 57% was eliminated in feces. Of the radioactivity present in urine, none was present as unchanged WR242511; five major radiolabeled metabolite peaks were resolved in urine by HPLC. The percentage of unchanged WR242511 eliminated in individual feces samples collected at 6-hr intervals after dosing with [^{14}C]WR242511 accounted for less than 0.5% of the dose. These results indicated that WR242511 was extensively metabolized by dogs.

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An effort to develop orally active, long-lasting anti-cyanide agents led to a interest in the para-aminophenones, in particular, PAHP (p-aminoheptanophenone). PAHP has been shown to produce methemoglobin at a level that corresponds to the level necessary for cyanide detoxification in animals. Theoretically, PAHP can be given on a 6-8 hour dosing schedule to maintain levels of methemoglobin between 5% and 8%. Methemoglobin levels below 10% are considered to be well tolerated in humans and animal species.

Only limited studies on the pharmacokinetics and metabolism of PAHP have been accomplished. The results of a study conducted by Marino, in which an i.v. dose of either 2 or 6 mg/kg of PAHP was administered to dogs, indicated that the elimination of PAHP was well described by a two compartment model with an apparent distribution half-life of about 20 min and a terminal half-life of about 4 hr[8]. In an oral bioavailability study conducted in our laboratory[9], peak blood levels of radioactivity were achieved in dogs within 45 to 90 min after oral administration of [14C]PAHP; thereafter, the dogs eliminated radioactivity from whole blood in two apparent phases with half-lives of approximately 0.9 hr and 37 hr. Similar half-life values were computed for dogs administered the same dose i.v. Following either route of administration, the levels of radioactivity in red blood cells increased with time after dosing such that beyond 12 hr post dose most of the radioactivity in blood was associated with the red blood cells. Highest concentrations of unchanged PAHP were observed in plasma immediately after administration of the i.v. dose and at 30 to 90 min after the oral dose. Peak concentrations of methemoglobin in blood were achieved at 2 to 3 hr after either an i.v. or oral dose. After oral administration of [14C]PAHP, the mean urinary elimination of radioactivity accounted for 59.2% of the dose and the fecal elimination, 27.3%. With i.v. dosing, mean values of 71.5% and 21.8% of the dose were eliminated in urine and feces, respectively. HPLC analyses indicated that no detectable amounts of unchanged PAHP were excreted in urine following either i.v. or oral administration of [14C]PAHP; most of the radioactivity in urine was associated with three major radiolabeled metabolites. Unchanged PAHP was found in feces following either oral or i.v. [14C]PAHP. In addition, two major radiolabeled metabolites, and several minor metabolites, were detected in feces.

In a bioavailability study conducted in our laboratories in rats[10], peak blood levels of radioactivity were achieved 45 min after oral administration of [14C]PAHP and 15 min after i.v. administration. With time after either oral or i.v. dosing, a greater percentage of the radioactivity in blood became associated with the red blood cells. Peak plasma concentrations of unchanged PAHP occurred 15 min after either oral or i.v. administration of [14C]PAHP. Methemoglobin concentrations in blood increased rapidly and reached peak levels 45 min after oral dosing and 15 min after i.v. dosing. For rats dosed orally, a mean value of 71.2% of the dose was eliminated in urine and 22.1% in feces. After i.v. dosing, the mean values for the urinary and fecal elimination of radioactivity were 79.3% and 13.7%, respectively. HPLC analyses indicated that no unchanged PAHP was eliminated in urine after either oral or i.v. administration; the majority of the radioactivity in urine was associated with three radioactive metabolites which had different retention times from the metabolites eliminated in dog urine. Unchanged PAHP was eliminated in feces after either oral or i.v. administration of [14C]PAHP. In addition, three major and several minor metabolites were detected in feces.

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Stene[11] investigated the metabolism of PAHP in the isolated perfused rat liver using uniformly labeled [^{14}C]PAHP and found that at least ten major radiolabeled metabolites were produced. Of these, one was identified as an N-hydroxy derivative of PAHP. The results of Stene's studies have raised an important concern relevant to the clinical development of PAHP. This concern is based on the potential toxicity known to be associated with N-hydroxylated aromatic amines. Investigations of the metabolism of the compound would appear to be imperative.

● **Anti-Cholinesterase Antidotes**

Poisoning by soman, an extremely toxic organophosphate anticholinesterase agent, is resistant to treatment with conventional acetylcholinesterase (AChE) oxime reactivators, including 2-PAM chloride (pyridine-2-aldoxime chloride). 2-PAM chloride is the pyridinium component of the U.S. Army's currently fielded medical protection against anticholinesterase agents. In an effort to improve survival from soman poisoning, Hagedorn and coworkers[12] synthesized a class of bis-pyridinium oximes known as H-oximes. The efficacy of one member of this class, HI-6, against soman-induced toxicity has been repeatedly confirmed in several different species in a multitude of in vitro and in vivo studies[13-17].

In a study of the mechanism of action of HI-6, Hamilton and Lundy[18] used soman and tabun, another organophosphorus compound, as HI-6 challenge agents. These investigators reported that HI-6 did not reactivate tabun-inhibited AChE in rat diaphragm, yet protected against a 2-5 x LD_{50} dose of nerve agent. In the same report, Hamilton and Lundy demonstrated that although soman-inhibited plasma AChe was not reactivated by HI-6 in Rhesus monkeys, the animals were protected from the lethal effects of a 5 x LD_{50} dose of soman. It is to be noted that unless carefully controlled, soman-inhibited AChE becomes nonresponsive to reactivation over time, a process called "aging."

Other mechanisms have been postulated to be possible contributors to the effectiveness of HI-6 against AChE agent lethality. Several studies have shown that H-oximes possess mild in vitro and in vivo antimuscarinic activity, either as a result of binding to a different site from that occupied by atropine or by altering the receptor itself[19,20]. Other biological effects of H-oximes include ganglionic blockade, effects on the neuromuscular junction and possible effects on channel opening. At present it is not clear whether these actions play a significant role in the efficacy of oximes against nerve agents.

The pharmacokinetics of HI-6 has been studied in several species. Simon and Briggs[21] investigated the disposition of HI-6 in rats. After i.v. administration of 20 mg/kg, plasma concentrations of HI-6 of about 140 μg/ml were achieved 3 min after dosing; the compound was subsequently eliminated with a half-life of approximately 65 min. The apparent volume of distribution of HI-6 was 380 ml/kg. After i.m. dosing, peak plasma levels of HI-6 were observed at about 15 min. Beyond this time, the compound was eliminated in a single phase with a half-life of 59 min. The volume of distribution of HI-6 after i.m. administration was calculated to be 370 ml/kg.

The distribution of HI-6 in rats following i.v. administration of [^{14}C]HI-6, labeled at the carbon of the carboxamide moiety, was studied by Ligtenstein and coworkers using autoradiography[22] . These investigators confirmed the penetration of radiolabel into the central nervous system. The gastrointestinal tract was found to be largely devoid of radioactivity but significant activity was detected in the kidneys, heart, liver, nose, bladder, testes and marrow-containing bone. Using similarly labeled HI-6, Lundy and coworkers[23] administered [^{14}C]HI-6 to groups of male and female rats and then determined the distribution of radiolabel using both autoradiographic and liquid scintillation spectrometric techniques. In both sexes, radioactivity levels were highest in kidney, followed in order by cartilage > plasma > liver > heart $\geq$ lung $\geq$ diaphragm > brain and spinal cord.

The metabolism of HI-6 in rats has been investigated by Ligtenstein and colleagues[24]. Using preparative HPLC, two metabolites were isolated from urine collected from rats administered 50 mg/kg of HI-6, i.v. Mass spectral, gas chromatographic, infrared, UV and NMR spectrometric analyses established that both metabolites contained 2-pyridone moieties. One metabolite had an intact pyridium-aldoxime moiety. The authors concluded that the excretion of unchanged HI-6 and the two metabolites did not provide for 100% mass balance, indicating that other unidentified metabolites were also formed.

Simon and Briggs[25] also studied the disposition of HI-6 in dogs after intravenous and intramuscular administration. Mean initial HI-6 plasma concentrations after i.v. administration of 20 mg/kg of HI-6 to seven dogs were about 93 μg/ml. The mean distribution half-life for the i.v. dose was determined to be 6.3 min and the elimination half-life, 48.2 min. After a 20 mg/kg i.m. dose, peak plasma concentrations of HI-6 of about 45 μg/ml were achieved approximately 21 min after dosing. The half-life of elimination of i.m. administered HI-6 was to determined to be similar to that obtained after i.v. dosing. From the areas under the plasma concentration versus time curves computed for HI-6 following either i.v. or i.m. administration, these investigators concluded that HI-6 was 100% bioavailable following i.m. administration.

Baggot and coworkers[26] determined the absorption and disposition kinetics of HI-6 in nine beagle dogs following administration of a single dose of 25 mg/kg by i.v., i.m. and oral routes. Following i.v. administration, HI-6 was rapidly eliminated, mainly by renal excretion; about 64% of the dose was excreted in urine as unchanged HI-6 within the first 24 hr after dosing. The half-life calculated for the elimination of HI-6 from plasma after i.v. dosing was about 49 min. This value was in agreement with that determined by Simon and Briggs[25]. After i.m. administration, HI-6 was rapidly absorbed and then eliminated from plasma with an apparent half-life of about 43 min. The fraction of the dose excreted in urine as unchanged HI-6 (about 58%) was similar to that determined after i.v. dosing. The systemic bioavailability of i.m. administered HI-6 was calculated to be approximately 90%. HI-6 was poorly absorbed following oral administration and eliminated from plasma with a half-life of about 72 min. In this study, no attempt was made to isolate and identify the metabolites of HI-6.

Eyer, Kawan and Ladstetter[27] reported the formation of cyanide in beagle dogs following i.v. administration of HI-6. They estimated that, for HI-6, 80% was eliminated unchanged, 15% was metabolized by non-cyanide routes and 4% was converted to cyanide.

Ecobichon and coworkers[28] compared the biotransformation of HI-6 in the rat, dog and rhesus monkey. Three degradation products were detected in the plasma of all three species. One product excreted in urine appeared to form spontaneously since it was detected in buffered solution used for stability studies. This product appeared to be the same as one of the metabolites isolated by Ligtenstein[24]. The second product was identified as a picolinic acid analogue of HI-6 and appeared to be metabolically formed in vivo. The third product remained identified.

In a study conducted in our laboratory[29], the pharmacokinetics and metabolism of [^{14}C]HI-6 dichloride were investigated in male Beagle dogs following administration of a dose of 30 mg/kg by i.m. injection. Following i.m. injection of [^{14}C]HI-6, radioactivity was rapidly absorbed, with a half-life of absorption of 0.04 hr, and peak levels of radioactivity were achieved in whole blood within 20 min after dosing. The elimination of radioactivity from whole blood appeared to occur in two phases with mean life-lives of 0.85 hr and 145.2 hr. Beyond about 4 hr after dosing, the levels of radioactivity in whole blood were higher than those in plasma, suggesting that radioactivity derived from [^{14}C]HI-6 was being sequestered in red blood cells with time after dosing. Plasma radioactivity data were best fit to a triexponential curve with a single absorption phase of about 0.07 hr and two elimination phases with half-lives of 0.86 hr and 29.3 hr. For four of six dogs, plasma concentrations of unchanged HI-6 declined in an apparent single phase with a mean half-life of about 0.94 hr. For two dogs, two phases of elimination of unchanged HI-6 of 0.31 hr and 1.3 hr were observed. Cyanide concentrations in red blood cells increased above the pre-dose levels within 6 min after administration of [^{14}C]HI-6, reached peak levels of approximately 270 to 350 ng/ml of RBC at 60-90 min after dosing and then declined to the approximate pre-dose levels within 8 hr after dosing. In dogs, a mean value of 84.1% of the dose was recovered in the combined collections of urine and feces within 7 to 14 days after dosing. Of this amount, a mean value of 80.3% of the dose was eliminated in urine and 4.1% in feces. The majority of the radioactivity recovered in urine was excreted within the first 12 hr after dosing. HPLC analyses of urine indicated that during the first 2 hr after administration of [^{14}C]HI-6, greater than 80% of the radioactivity excreted in urine was unchanged [^{14}C]HI-6. In addition to unchanged HI-6, at least five radiolabeled metabolites were detected in urine.

As noted previously, the focus of work during this past year of the contract has been to isolate and identify the urinary and fecal metabolites of HI-6, WR242511 and PAHP which were excreted in samples collected during the pharmacokinetic studies completed in our laboratory. A description of the methods used and results obtained during the 1995 contract year is provided below.

II. METHODS

A. Test Compounds

Investigations were conducted to isolate and identify the metabolites of the following test compounds:

●**HI-6**, WR249655, 1-(2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino)-2-oxapropanedichloride. The compound used during the study was radiolabeled with [^{14}C] in the 2-hydroxyiminomethyl group. The structure of HI-6 is shown in Figure 1.

●**PAHP**, para-aminoheptanophenone. The carbons in the phenone ring were universally labeled. The structure of PAHP is shown in Figure 2.

●**WR242511**, 8-[(4-amino-1-methylbutyl)amino]-5-(1-hexoxy)6-methoxy-4-methyl-quinoline (DL)-tartrate. The compound used during the study was labeled with [^{14}C] in the 4-carbon of the quinoline ring. The structure of WR242511 is shown in Figure 3.

B. Analytical Equipment

HPLC analyses were accomplished using a Waters Associates (Milford, MA) liquid chromatographic system equipped with two Model 510 pumps, a Model 680 automated gradient controller, a Model 712 WISP™ automatic sample injector, a System Gold™ Module 168 diode array detector (Beckman Instruments, Inc., San Ramon, CA) and a Flo-One/Beta Series A-500 radiochromatography detector (Radiomatic Instruments and Chemical Company, Inc., Meriden, CT).

During individual HPLC analyses, real time absorbance spectra were obtained on eluting sample components using the diode array detector. For these, absorbance was measured as a function of wavelength and time simultaneously.

Mass spectra, in the chemical (CI) ionization mode, were obtained on a Sciex API III triple-quadrapole mass spectrometer (Perkin-Elmer Corp., Norwalk, CT). Prior to CI mass spectral analysis, each metabolite or reference compound was dissolved in acetonitrile:water (50:50, v:v). The individual solutions were injected into a 0.009 ml/min flow of the same solvent containing 2 mM ammonium acetate. This flow was provided by a syringe pump (Harvard Apparatus Inc., South Natick, MA). Ionization was performed by an ion spray (electrospray) interface.

The migration of radioactive sample components separated by TLC was assessed using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA) equipped with a Hewlett-Packard (Palo Alto, CA) Vectra QS/165 computer and Hewlett-Packard Laser Jet series III printer.

Radioactive samples were counted in a Packard Tri-Carb 1900TR or 1900CA liquid scintillation analyzer (Packard Instrument Co., Inc., Downers Grove, IL).

C. Isolation and Identification of Urinary Metabolites of HI-6

1. Isolation of HI-6 Metabolites from Urine

Urine samples for metabolite isolation and identification were obtained from male Beagle dogs at various times after i.m. administration of 30 mg/kg of [^{14}C]HI-6 dichloride[29].

Individual metabolite peaks in the samples were isolated by HPLC following repetitive injection of urine under the following conditions of analysis:

Column:	Nucleosil C18, 250 x 4 mm (Keystone Scientific Inc., Bellfonte, PA).
Elution:	Linear gradient; 0% B to 100% B in 25 min with a 15 min hold at final conditions
	Solvent A: 5% Acetonitrile containing 0.001 M 1-octane sulfonic acid and 1% acetic acid
	Solvent B: 20% Acetonitrile containing 0.001 M 1-octane sulfonic acid and 1% acetic acid
Flow rate:	1 ml/min
Detection:	260 nm; on-line radioactivity monitor

Radioactive components which eluted from the column were collected separately as they eluted from the column. Each collected peak was identified by its elution time. The combined collections of each metabolite peak were placed under a stream of nitrogen to evaporate organic solvent present and then lyophilized to dryness. Individual metabolite peaks were then further purified as described below in Section II.C.2.

The presence of an unlabeled metabolite of HI-6 was determined by comparison of the UV chromatographic profiles of urine samples collected from dogs before and after administration of [^{14}C]HI-6. Quantitation of the unlabeled metabolite was based on the molar extinction coefficient of HI-6.

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2.   Purification and Identification of HI-6 Metabolites

a.   22 min metabolite peak

The lyophilized sample was mixed with 2 ml of acetonitrile to affect precipitation of the octane sulfonic acid which had been present in the HPLC mobile phase. After centrifugation, the organic supernate was removed and evaporated to dryness under a stream of nitrogen. The sample was reconstituted in water and then re-purified by HPLC, under gradient elution conditions, using a Nucleosil C18 column. Solvent A consisted of deionized water and solvent B contained acetonitrile:water (17:83, v/v). Elution was accomplished with a linear gradient, 0% to 100% B in 20 min with a 10 min hold at final conditions. The flow rate was 1 ml/min. The purified metabolite sample was lyophilized to dryness and stored at -70°C prior to mass spectral analysis.

b.   19 min metabolite peak

The lyophilized sample was "de-salted" with acetonitrile, as described above for the 22 min peak. After reconstitution in deionized water, the metabolite peak was re-purified by HPLC using a Nucleosil C18 column (as described above) and a mobile phase of acetonitrile:water (10:90, v/v). Elution was isocratic at a flow rate of 1 ml/min. The purified metabolite sample was lyophilized to dryness and stored at -70°C prior to mass spectral analysis.

c.   14 min metabolite peak

After lyophilization, separate collections of the 14 min peak were reconstituted in either deionized water, acetonitrile or methanol. Radioassay data obtained after lyophilization of this metabolite suggested that at least a portion of the metabolite peak was volatile and was being lost during the lyophilization process. To verify this, six equal portions (0.5 ml) of the 14 min metabolite peak, as collected from the HPLC column, were placed in scintillation vials. Two portions were immediately mixed with Soluene 350 tissue solubilizer and scintillation cocktail; the samples then were radioassayed. These were termed "wet" samples. In addition, four portions of the 14 min metabolite peak were evaporated to dryness under a stream of nitrogen, yielding what were termed "dry" samples. To two dry samples, 0.5 ml of Soluene 350 was added; to the other two dry samples, 0.5 ml of water was added. The dry samples containing reconstituting agent were placed in a shaker rack at 37°C and agitated overnight (approximately 16 hr). Scintillation cocktail was then added to the vials and the samples were radioassayed.

d.   2-4 min metabolite peak

The lyophilized sample was reconstituted in deionized water, radioassayed to check the recovery of radioactivity and then subjected to various procedures in an attempt to increase the retention time of this very polar metabolite peak. These procedures included: a.) modification of the conditions of HPLC analysis described in Section II.C.1 by reduction of the organic solvent strength of the mobile phase and by elimination

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of the ion pairing agent. b.) treatment of the metabolite peak with diazomethane to possibly increase the retention time of the peak components through the formation of less polar methylated derivatives. The methylation procedure was as follows: to a lyophilized portion of the metabolite peak, ethereal diazomethane was added until a persistent yellow color was obtained. The sample was maintained at room temperature for at least 15 min, subsequently evaporated to dryness under a stream of nitrogen and assayed by HPLC.

Since neither of the above two procedures were effective in altering the retention time of the 2-4 min metabolite peak, the sample was subsequently analyzed by thin layer chromatography (TLC) as follows: a portion of the metabolite peak was applied to a Silica Gel 60 TLC plate (250 micron thickness, Whatman, Inc., Clifton, NJ) and the plate was developed in acetone:methanol (2:1, v:v). After drying, UV absorbing components in the sample were detected by viewing the plate under a UV light. Radioactivity on the plate was quantitated using a radioactivity imager.

D. Isolation and Identification of Metabolites of PAHP Excreted in Dog Urine

1. Isolation of PAHP Metabolites from Dog Urine

Urine samples for metabolite isolation and identification were obtained from male Beagle dogs at 0-6 hr after either oral or i.v. administration of 7 mg/kg of [^{14}C]PAHP[9].

Four radioactive metabolite peaks in dog urine were isolated by HPLC following repetitive injection of urine under the following conditions of analysis:

Column:	Nucleosil C18, 250 X 10 mm (Keystone Scientific, Inc., Bellfonte, PA).
Elution:	Linear gradient; 0% B to 100% B in 40 min
	Solvent A: 0.15% phosphoric acid
	Solvent B: 50% acetonitrile containing 0.15% phosphoric acid
Flow rate:	4 ml/min
Detection:	316 nm; on-line radioactivity monitor

Each metabolite peak was collected separately as it eluted from the column and was identified by its elution time. The combined collections of each metabolite were placed under a stream of nitrogen to evaporate organic solvent present and then lyophilized to dryness. Individual metabolites were then further purified as described below in Section II.D.2.

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2.   Purification and Identification of PAHP Metabolites from Dog Urine

a.   26 min metabolite peak

The lyophilized sample was resuspended in 100% methanol and then analyzed by HPLC under the following conditions of analysis:

Column:	As above for initial metabolite isolation
Elution:	Isocratic 40% acetonitrile in water
Flow rate:	3 ml/min
Detection:	313 nm; on-line radioactivity monitor

Upon analysis of the 26 min metabolite peak under the above HPLC conditions, the metabolite peak eluted as two separate radioactive components with retention times of approximately 7 min and 14 min. These peaks were termed "M1" (7 min peak) and "M2" (14 min peak). To obtain purified fractions of M1 and M2, the 26 min metabolite peak was repeatedly chromatographed on HPLC under the isocratic conditions described above and the M1 and M2 peaks were collected separately as they eluted off the column. The collected fractions were dried under nitrogen to remove acetonitrile and then lyophilized to dryness. The purified samples of M1 and M2 were stored at -70°C prior to mass spectral analysis.

b.   11 min metabolite peak

The lyophilized 11 min metabolite peak, which was termed "M4", was resuspended in methanol and subjected to further purification by HPLC under the following conditions of analysis:

Column:	As above for initial metabolite isolation
Elution:	Isocratic 5% Acetonitrile in water
Flow rate:	3 ml/min
Detection:	254 nm; on-line radioactivity monitor

The collected fractions of M4 were evaporated under nitrogen and then lyophilized to dryness. The purified sample was stored at -70°C prior to mass spectral analysis.

c. 9 min metabolite peak

The lyophilized 9 min metabolite peak, which was termed "M3", was resuspended in methanol and re-chromatographed under the HPLC conditions used for the initial isolation of the metabolite (See Section II.D.1) in order to determine its purity.

E. Isolation and Identification of Metabolites of PAHP Excreted in Rat Urine

1. Isolation of PAHP Metabolites from Rat Urine

Urine samples for metabolite isolation and identification were obtained from male Sprague-Dawley rats at 0-6 hr after i.v. administration of 7 mg/kg of [^{14}C]PAHP[10]. Urine samples collected from four rats were pooled, lyophilized and reconstituted in water to affect a 3-fold concentration of the sample prior to metabolite isolation.

Two radiolabeled metabolite peaks, which eluted at retention times of 21 min and 26 min, were isolated from rat urine by HPLC following repetitive injection of urine using the same assay conditions and procedures as used for the isolation of metabolites from dog urine (See Section II.D.1). The combined collections of each metabolite were placed under a nitrogen stream to remove the organic solvent present and then lyophilized to dryness. The two metabolites were further purified as described in the following section.

2. Purification and Identification of PAHP Metabolites in Rat Urine

a. 21 min metabolite peak

The lyophilized 21 min metabolite peak, which was termed "M5", was resuspended in methanol and an attempt was made to further purify the metabolite by HPLC under the following conditions:

Column:	Nucleosil C18, 250 x 10 mm (Keystone Scientific, Inc.)
Elution:	Isocratic 20% acetonitrile in water
Flow rate:	3 ml/min
Detection:	254 nm; on-line radioactivity monitor

For re-purification, portions of the metabolite sample were successively injected into the column and the peak identified as M5 was collected, evaporated, lyophilized to dryness and stored under a nitrogen atmosphere. The purity of the re-purified M5 then was determined by HPLC under the above conditions, with the exception that the size of the Nucleosil C18 column used was 250 x 4 mm.

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b. 26 min metabolite peak

The lyophilized 26 min metabolite, which was termed "M6", was resuspended in methanol and further purified by HPLC under the following conditions:

Column: Nucleosil C18, 250 x 10 mm (Keystone Scientific, Inc.)

Elution: Isocratic
                 30% acetonitrile in water

Flow rate: 3 ml/min

Detection: 254 nm; on-line radioactivity monitor

The purification procedure was accomplished as described above for M5. The purified sample of M6 was stored at -70°C prior to mass spectral analysis.

F.   Isolation and Identification of Metabolites of PAHP Excreted in Dog Feces

1.   Isolation of PAHP Metabolites from Dog Feces

Feces samples for metabolite isolation and identification were obtained from male Beagle dogs at 12-24 hr after either oral or i.v. administration of 7 mg/kg of [^{14}C]PAHP[9]. Prior to metabolite isolation, extracts of feces were prepared in methanol. For this, fecal homogenates (previously prepared in water) were lyophilized to dryness and then extracted twice with approximately 2 volumes of methanol. The methanol extract was concentrated under a stream of nitrogen and portions of the extract were repetitively injected onto an HPLC under the following conditions:

Column: Nucleosil C18, 250 X 10 mm (Keystone Scientific, Inc., Bellfonte, PA).

Elution: Linear gradient; 0% B to 100% B in 40 min

                 Solvent A: 0.15% phosphoric acid

                 Solvent B: 50% acetonitrile containing 0.15% phosphoric acid

Flow rate: 4 ml/min

Detection: 316 nm; on-line radioactivity monitor

Individual metabolite peaks were collected separately as they eluted from the column and were neutralized with 1 M ammonium hydroxide. The combined collections of each metabolite were placed under

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a stream of nitrogen to evaporate the acetonitrile in the solvent and then lyophilized to dryness. The extracts were analyzed by HPLC under the conditions of analysis described above with the exception that the Nucleosil C18 column used was analytical size (250 x 4 mm).

G.   Isolation and Identification of Metabolites of PAHP Excreted in Rat Feces

1.   Isolation of PAHP Metabolites from Rat Feces

Feces samples for metabolite isolation and identification were obtained from male Sprague-Dawley rats at 6-24 hr after either oral or i.v. administration of 7 mg/kg of [^{14}C]PAHP[10]. Prior to metabolite isolation, extracts of rat feces were prepared in methanol as described above for dog feces. Extracts then were analyzed by HPLC under the conditions described above for dog feces.

H.   Isolation and Identification of Metabolites of WR242511

1.   Isolation of WR242511 Metabolites from Dog Urine

Two chromatographic methods, HPLC and TLC, were investigated for utility in achieving resolution and purification of the radiolabeled metabolites of WR242511 excreted in dog urine.

a.   HPLC methodology

Urine samples for metabolite isolation and identification were obtained from male dogs at 6 or 12-hr time intervals through 24 hr after oral administration of 7 mg/kg of WR242511[7]. Samples were concentrated by lyophilization and resuspended in deionized water prior to chromatographic analysis.

Four radiolabeled metabolites of WR242511, with retention times of 9, 13, 28 and 34 min, were isolated from dog urine using HPLC with the following conditions of analysis:

Column:	Spherisorb CN, 5 micron, 250 x 4.6 mm or 250 x 10 mm
Elution:	Linear gradient; 0% B to 100% B in 40 min
	Solvent A: 10 mM ammonium acetate, pH 6.0, with 1 mM EDTA
	Solvent B: acetonitrile: 20 mM ammonium acetate containing 1 mM EDTA (50:50; v/v).
Flow rate:	1 or 3 ml/min
Detection:	262 nm and on-line radioactivity monitor

The radiolabeled metabolites of interest which eluted from the column were collected separately as they eluted from the column. Metabolites were initially identified by retention time. The combined collections of each metabolite were placed under a stream of nitrogen to evaporate the organic solvent in the sample and then lyophilized to dryness.

1.   34 min metabolite peak

The lyophilized sample, which was termed "WR4," was resuspended in acetonitrile:water (1:1,v/v) and the purity of the sample was ascertained by HPLC using the assay conditions described above for the initial collection of the metabolite. WR4 was then partially desalted by solid phase adsorption on a SepPak C18 column (Waters Associates, Bedford, MA) and subsequently analyzed by mass spectrometry.

2.   28 min metabolite peak

The lyophilized radiolabeled 28 min metabolite peak, termed "WR3," was resuspended in acetonitrile:water (1:1,v/v) and further purified by HPLC under the conditions described above for the initial isolation of the metabolite from urine. The re-purified WR3 sample was evaporated under nitrogen, lyophilized to dryness and then analyzed by mass spectrometry.

3.   9 min and 13 min metabolite peaks

The 9 min (WR1) and 13 min (WR2) radiolabeled metabolites of WR242511 which were collected by HPLC contained a significant amount of endogenous UV absorbing impurities. Work on the further purification of these metabolites is pending.

b.   TLC methodology

Urine samples collected from dogs administered [14C]WR242511 were applied to Silica Gel 60 TLC plates. Acetone and various combinations of acetone:methanol and methanol:chloroform were investigated for their ability to effect the migration of the radiolabeled components in the samples. After development, the plates were viewed under UV light; radioactivity on the plates was located and quantitated using a TLC radioscanner. Radioactive areas of interest were eluted from the gel with methanol, concentrated under a stream of nitrogen and subsequently analyzed by HPLC under the conditions described in Section II.H.1.a.

2.   Isolation of Metabolites of WR242511 from Red Blood Cells

Red blood cells (RBCs) collected from 48 to 144 hr after i.v. administration of 7 mg/kg of [14C]WR242511 to dogs[7] were pooled and lyophilized to dryness. The lyophilized samples then were extracted successively with methylene chloride, acetone, methanol and water in order to achieve fractionation of the radioactivity in the sample based on the polarity of the radiolabeled component(s). Portions of each RBC

extract were radioassayed. The extracts were either evaporated to dryness under a stream of nitrogen (organic extracts) or lyophilized (aqueous extract) and stored for subsequent HPLC analysis.

III. RESULTS

A.   Identification of the Urinary Metabolites of HI-6

In our 1994 Annual Report, we reported quantitative data for six radiolabeled peaks which were resolved during the HPLC analysis of urine collected from dogs administered an i.m. dose of [^{14}C]HI-6. A representative HPLC chromatogram which shows the metabolite profile of these samples is presented in Figure 4. In efforts completed during this past contract year, an additional unlabeled urinary metabolite of HI-6 was isolated and tentatively identified. A revised summary of the urinary metabolites of HI-6, which includes data for this previously undetected metabolite, is presented in Table 1.

The results obtained during the isolation and identification of the urinary metabolites of [^{14}C]HI-6 which were excreted by dogs is provided below.

1. 19 min metabolite

Upon HPLC analysis, the 19 min metabolite of [^{14}C]HI-6, which retained the [^{14}C]label, had the same retention time as authentic pyridine-2-aldoxime. The UV absorbance spectrum of the metabolite matched that of authentic pyridine-2-aldoxime (Figure 5). Chemical ionization mass spectrometry (CIMS) established that the spectrum of the 19 min metabolite was essentially identical to that of authentic pyridine-2-aldoxime (Figure 6). Protonated ions at m/z 123, 105 and 79 were obtained in the CIMS spectrum. The ion m/z 123 indicated protonated pyridine-2-aldoxime, and m/z 105 a protonated dehydrated form of the compound. These results indicated that the 19 min metabolite of HI-6 was pyridine-2-aldoxime. The structure of this metabolite is shown in Figure 7.

2. 22 min metabolite

Initial attempts to isolate and identify the 22 min metabolite of [^{14}C]HI-6 were based on the assumption that the large UV absorbing peak that eluted at about 22 min under the gradient conditions of HPLC analysis used was associated with the radioactivity which also eluted at essentially the same retention time. Subsequent chromatographic analyses indicated, however, that the 22 min metabolite peak was a separate entity from the radioactive peak. Thus, the 22 min metabolite did not contain a radiolabeled carbon.

The UV absorbance spectrum of the 22 min metabolite is shown in Figure 8. A spectrum of HI-6, obtained under similar chromatographic conditions, is also included in the figure. The 22 min metabolite exhibited an absorbance maximum at 267 nm which was slightly shifted from the maximum at 273 nm exhibited by HI-6. In addition, the 22 min metabolite displayed decreased absorbance, relative to HI-6, at 304

nm, indicating the absence of the oxime group. Upon treatment of urine with acid (0.3 N HCl, 80-100°C for 15 min), the 22 min metabolite was partially degraded to more polar degradation products (which were not characterized). Mass spectral analysis (chemical ionization) of the metabolite showed predominant protonated ions at m/z 108, 138 and 260 (Figure 9). The ion at m/z 108 presumably stemmed from 1-methyl-2-pyridone and that at m/z 138 from 1-methyl-4-carboxamido-pyridine. The m/z 260 indicated the presence of a ion containing an oxydimethylene bridge and may have been a dehydride of the intact metabolite. This metabolite has been tentatively identified as a pyridone derivative of HI-6 and its propose structure is shown in Figure 7.

3. 14 min metabolite

Several attempts were made to isolate and identify the 14 min radiolabeled metabolite of HI-6, which accounted for between 5.5% and 5.7% of the dose in some urine samples. After collection of this metabolite by HPLC, substantial (greater than 85%) loss of radioactivity was noted following lyophilization of the sample and reconstitution in either acetonitrile, methanol or water. Initially, the loss of radioactivity was assumed to result from adherence of the sample to the lyophilizing vessel. Subsequent experiments in which the metabolite peak, as collected from the HPLC, was placed in a scintillation vial, evaporated to dryness, resuspended in Soluene 350 (a strong digesting agent) and then radioassayed indicated that the metabolite was volatile. This finding curtailed further attempts to isolate and identify the 14 min metabolite.

4. 2-4 min metabolite peak

The results of the initial HPLC analyses of urine indicated that the 2-4 min metabolite peak probably consisted of more than one radiolabeled component. Since this peak was very poorly retained under the conditions of HPLC analysis used, several steps were taken in an effort to increase the retention time of the peak in order to effect greater resolution of the components in the peak. Modification of the mobile phase by decreasing the organic solvent concentration and elimination of the ion pairing agent were not effective in producing an increase in retention time for any radiolabeled components in the peak. In addition, the isolated metabolite peak was treated with diazomethane in an effort to produce less polar methylated derivatives which would be more readily retained on the column. However, diazomethane treatment had no effect on the retention time of any radioactive components in the peak.

The 2-4 min metabolite peak was subsequently analyzed by thin layer chromatography using Silica Gel 60 plates developed in acetone: methanol (2:1, v:v). Under these conditions, three radioactive components were resolved in the peak. The majority of the radioactivity in the metabolite peak was associated with two components which each contained 40-45% of the total radioactivity in the peak. These two components had approximate R_fs of 0.75 and 0.20. The radioactive component at R_f 0.75 migrated on the plate with a characteristic "W" shape and, thus, was termed "Peak W." The other peak (R_f 0.20) was termed "Peak Y." The remainder of the radioactivity in the 2-4 min peak remained at the origin ("Peak X").

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Several attempts were made to isolate components W, X and Y from the TLC plates and obtain a sufficiently pure quantity for mass spectral analysis. These attempts were not successful.

Based on the structure of HI-6 and the position of the radiolabel, it was reasoned that picolinic acid was a possible radiolabeled metabolite produced from [^{14}C]HI-6 and that it may be a component of the 2-4 min metabolite peak. As such, authentic picolinic acid was assayed by both HPLC and TLC under the conditions described in Sections II.C.1 and II.C.2.d, respectively. Upon HPLC analysis, authentic picolinic acid had a retention time of 3.2 min. On TLC, the R_f of picolinic acid was similar to that of Peak Y. Thus, it is possible that one of the components (Y) of the 2-4 min metabolite peak was picolinic acid.

No further efforts were made to identify the components of the 2-4 min metabolite peak.

Significance. Both chromatographic and mass spectral analyses established that the major radiolabeled metabolite of [^{14}C]HI-6 excreted in urine was pyridine-2-aldoxime (19 min metabolite). This metabolite has not previously been identified in either urine or plasma samples collected from experimental animals and humans administered HI-6. Ladstetter[30] has proposed that pyridine-2-aldoxime is a metabolic product of HI-6 but did not detect this metabolite after HI-6 administration to dogs. In his study, however, the position of the radiolabel was in the carboximide moiety; any pyridine-2-aldoxime formed would not have been radiolabeled and, thus, not easily detectable. Indirect evidence for the formation of pyridine-2-aldoxime is found in the fact that, in Ladstetter's study, 3% of the dose was recovered as isonicotinamide, the other half of the cleaved HI-6 molecule. Based on a separate study conducted in one dog with pyridine-2-aldoxime, Ladstetter[30] concluded that pyridine-2-aldoxime serves only as an intermediate metabolite and is rapidly metabolized via either glucuronidation reactions or other metabolic pathways. We have found no evidence for the presence of a radiolabeled glucuronide conjugate of HI-6 or its metabolites in urine.

The second urinary metabolite of HI-6 we identified appeared to be the same as that previously found by Ecobichon and coworkers[28] in rat, dog and monkey urine and plasma (metabolite II), by Ligtenstein and colleagues[24] in rat urine (metabolite B), and by Ladstetter[30] in dog urine. This metabolite has been identified by these investigators as 2-pyridone-1-yl-4'-
aminocarbonyl-1,1'-oxydimethylene pyridine. The production of this bridged pyridone metabolite is consistent with the increased blood cyanide concentrations observed by us[29] and Ladstetter[30] after HI-6 administration.

Ecobichon and colleagues[28] reported that rats, dogs and monkeys not only metabolized HI-6 to the pyridone metabolite but also to a picolinic acid analogue. Other investigators, however, have not confirmed these results and have reported the isolation of other metabolites of HI-6. In addition to metabolite B described above, a second metabolite isolated from rat urine by Ligtenstein[24] was structurally similar to HI-6 except that the 2'-ring was a pyridone. Ladstetter[30] identified a deaminated product of HI-6 in dog urine which was also dissimilar to Ecobichon's pyridone product. These reported differences in the metabolites of HI-6 may represent species differences in metabolism. Alternatively, with the exception of Ladstetter's study, unlabeled HI-6 was administered, making metabolite identification and mass balance determinations difficult, if not impossible. While we were unable to identity either of these three reported metabolites of HI-6, it is possible

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that the 22 min radiolabeled metabolite, whose retention time was consist with a bis-pyridinium or pyridone structure, represented one of these three metabolites. It would seem likely that more definitive information on the metabolism of HI-6 would be gained during metabolic studies by the administration of a formulation of HI-6 radiolabeled in an appropriate moiety of both pyridium rings.

Although, in this study, no direct structural information was obtained on the radiolabeled metabolites W, X and Y which comprised the 2-4 min metabolite peak resolved on HPLC, limited information obtained during the study provides some basis for postulating possible identities of these metabolites. Since the 22 min metabolite lacked the radiolabeled aldoxime moiety of HI-6, either peak W, X or Y may have been the cleaved radiolabeled aldoxime moiety or fragments of this moiety. In addition, the results of HPLC and TLC analyses indicated that the chromatographic behavior of picolinic acid, a known metabolite of pyridine-2-aldoxime, was similar to that of Peak Y.

Based on the results obtained during our study and published literature reports, we have proposed metabolic scheme for the metabolism of HI-6 which is presented Figure 10.

B.   Identification of the Metabolites of PAHP

1.   Identification of the Urinary Metabolites of PAHP Excreted by Dogs

A representative chromatographic profile obtained during the initial HPLC analyses of urine samples collected from dogs administered [^{14}C]PAHP is shown in Figure 11. The metabolite profile in urine was the qualitatively and quantitatively similar following either oral (Tables 2 and 3) or i.v (Tables 4 and 5) administration of [^{14}C]PAHP. Unchanged PAHP was not detected following either route of administration. The majority of the radioactivity was associated with three radiolabeled metabolite peaks which eluted at retention times of 9 min, 11 min and 26 min. During the isolation of the urinary metabolites, the 26 min peak was found to consist of two metabolites which were termed M1 and M2.

Work which was conducted during the past year of the contract to isolate and identify these metabolites of [^{14}C]PAHP is described below.

a.   M1 and M2 (26 min metabolites)

The UV absorbance spectrum of M1 indicated this radiolabeled metabolite of [^{14}C]PAHP had absorbance maxima at 241 and 327 nm. In addition, it displayed an absorbance maximum near 292 nm which appeared as a "shoulder" on the main 327 nm absorbance peak (Figure 12). By comparison, PAHP displayed wavelength maxima at 237 and 316 nm (Figure 12). Upon mass spectral analysis (chemical ionization) of M1, predominant protonated ions were observed at m/z 110, 136 and 238 (Figure 13). The ion at m/z 110 presumably stemmed from hydroxyaniline. The m/z 238, which was the major molecular species, indicated

that PAHP contained two added hydroxy groups. We have proposed that M1 is 6-[4'-hydroxyaminobenzoyl]hexanol. The proposed structure of M1 is shown in Figure 14.

The UV absorbance spectrum obtained for the urinary metabolite M2 is shown in Figure 15. The UV spectrum of this radiolabeled metabolite of PAHP was very similar to that of M1. Mass spectral analysis of M2 in the chemical ionization mode indicated predominant protonated ions at m/z 266 and 288 (Figure 16). The m/z 288 indicated the presence of a sodium molecule on m/z 266. The m/z 266 indicated M2 contained a methyl group which was not present on M1. The proposed structure of M2 is shown in Figure 14.

b.   M4 (11 min metabolite)

M4 displayed an absorbance maximum at 292 nm. Chemical ionization mass spectral analysis of M4 yielded results which were inconclusive. Further efforts are underway to identify this urinary metabolite eliminated by dogs.

c.   M3 (9 min metabolite)

Two attempts were made to purify the radiolabeled urinary metabolite, M3, for mass spectral analysis. The metabolite was isolated from dog urine by semi-preparative chromatography, evaporated and lyophilized to dryness and then re-analyzed by HPLC. The results of the HPLC analyses of the semi-purified metabolite indicated the presence of three radiolabeled components in the sample, none of which had the retention time (9 min) of the M3 peak initially collected by HPLC. These results suggested that M3 had degraded during the purification process. Further work is in progress to isolate and identify this metabolite.

2.   Identification of the Urinary Metabolites of PAHP Excreted by Rats

A representative chromatogram obtained during the HPLC analyses of urine collected from a rat administered [14C]PAHP is shown in Figure 17. In rats, the metabolite profile of urine samples collected from animals administered [14C]PAHP was different from the profile obtained for dog urine. Two major radiolabeled metabolites, which eluted at retention times of 21 min and 26 min, were resolved in rat urine; unchanged PAHP was not detected. The amount of each radiolabeled metabolite eliminated in urine was similar following either oral (Tables 6 and 7) or i.v. (Tables 8 and 9) administration of [14C]PAHP. The results of the efforts which have been completed to date to identify these urinary metabolites are described below.

a.   M5 (21 min metabolite)

The UV absorbance spectrum of the radiolabeled 21 min metabolite of PAHP, termed M5, is shown in Figure 18. The compound displayed one absorbance maximum at 327 nm and another near 295 nm, which appeared as a "shoulder" on the 327 nm peak. After the initial isolation of M5 from rat urine, an attempt was made to further purify this metabolite by HPLC. The initial attempt to obtain a

chromatographically pure sample of M5 for subsequent mass spectral analysis was not successful in that HPLC analyses indicated the final product contained radioactive components other than M5. Further experiments are planned to identify this metabolite.

b.   M6 (26 min metabolite)

Under identical conditions of HPLC analysis, the retention time of this radiolabeled urinary metabolite excreted by rats was similar to that of the M1/M2 peak which was isolated in dog urine; however, UV absorbance spectra indicated that the rat urinary metabolite, M6, was not the same as the dog urinary metabolites, M1 and M2. M6 displayed a single absorption maximum at 288 nm (Figure 18). Mass spectral analysis (chemical ionization) of M6 showed a predominant protonated ion at m/z 250 (Figure 19). This was confirmed by MS/MS analysis of the metabolite. These results suggested that M6 contained a methyl-hexano group that was not present on PAHP. The proposed structure of M6 is shown in Figure 20.

3.   Identification of the Fecal Metabolites of PAHP Excreted by Dogs

A representative chromatogram obtained during the HPLC analyses of feces collected from dogs administered [^{14}C]PAHP is shown in Figure 21. In feces, the majority of the radioactivity was associated with intact PAHP and two radiolabeled metabolites which had retention times of 24 min and 42 min; several minor radiolabeled peaks also were resolved. A difference was observed in the extent of metabolism of [^{14}C]PAHP following oral and i.v administration of the compound. In dogs given the oral dose, a higher percentage of the dose was eliminated as unchanged PAHP (Tables 10 and 11). Thus, a higher percentage of the dose was eliminated as metabolites in dogs administered the i.v. dose (Tables 12 and 13). The predominant fecal metabolite eluted at the same retention time (26 min) as the urinary metabolites M1 and M2. UV absorbance spectra obtained to date indicate that this fecal metabolite is different from both M1 and M2. Further work on the isolation and identification of the radiolabeled fecal metabolites excreted by dogs is currently in progress.

4.   Identification of the Fecal Metabolites of PAHP Excreted by Rats

A representative chromatogram obtained during the HPLC analyses of feces collected from rats administered [^{14}C]PAHP is shown in Figure 22. The elution pattern of radioactivity in feces collected from rats dosed with [^{14}C]PAHP was qualitatively similar to that in feces collected from dogs similarly dosed (Tables 14-17). Among rats administered the oral dose, considerable variation was observed in the amount of radioactivity eliminated as unchanged PAHP. This may have been related to the transit time of the dose through the gastrointestinal track of the individual animals. Efforts are ongoing to isolate and identify the major radiolabeled metabolites of PAHP in rat feces and to determine whether they are the same metabolites which are eliminated by dogs.

Significance.   Little is known about the metabolism of the para-aminophenones. Evidence gathered to date indicates that these compounds do not act directly to produce methemoglobin but that a metabolite of

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the parent compound is involved in the process. One of the proposed metabolites implicated in this process is an N-hydroxylated derivative, para-hydroxylaminophenone, which belongs to a class of compounds known to be associated with toxicity.

The results of mass spectral data obtained during our investigations indicate that dogs metabolize PAHP to one or more hydroxylated derivatives (identified as metabolites M1 and M2) which are excreted in urine, and possibly feces. We have proposed that, in dogs, the amino group in the para position of the phenone ring is one site of hydroxylation of the compound; it is anticipated that confirmation of this will be obtained from NMR analyses of the isolated metabolites. The toxicological threat posed by the formation of these metabolites most likely will be a function of their rate of formation and their rate of elimination and/or metabolism. The data obtained in our laboratory indicate that dogs eliminate 15 to 20% of a dose of PAHP as the proposed para-hydroxylated metabolites, M1 and M2. In contrast, to date, we have not found evidence for the presence of these metabolites in urine obtained from rats administered PAHP. These latter results suggest that a species difference in the metabolism of PAHP may exist. Thus, from a clinical standpoint, it will be important to determine to what extent and by what pathway humans metabolize PAHP.

C.   Identification of the Metabolites of WR242511

1.   Identification of the Urinary Metabolites of WR242511

In investigations completed the previous contract year, five major radiolabeled peaks, with retention times of 4, 9, 13, 28 and 34 min, were resolved during the HPLC analyses of urine collected from dogs administered [^{14}C]WR242511; no unchanged WR242511 was detected in urine (Figure 23). During this past year of the contract, efforts were focused on isolating and identifying these metabolites. In addition, since a metabolite of WR242511, which is apparently sequestered in RBCs, has been implicated in the production of methemoglobinemia following WR242511 administration, it was of interest to investigate the nature of the radioactivity previously detected in RBCs.

Isolation of the urinary metabolites of WR242511 has been hampered by two major difficulties: the low levels of each metabolite eliminated in urine and the inability to develop a chromatographic system that would effect adequate retention and baseline resolution of the metabolites. To date, mass spectral analyses have been conducted on two urinary metabolites of WR242511, the 28 min (WR3) and 34 min (WR4) peaks, which have been isolated by HPLC.

a.   WR4 (34 min metabolite peak)

Chemical ionization mass spectral analysis of the radiolabeled metabolite WR4 indicated that the sample submitted for analysis contained high levels of extraneous protonated components with a molecular weight of 500 and higher. Subsequent experiments established that these components were derived from the EDTA which was initially added to the HPLC mobile phase. In addition to these contaminants, the mass

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spectra of WR4 showed protonated ions at m/z 155, 211, 239 and 271; the negative mode (M-H)⁻ spectrum showed a predominant ion at m/z 209 (Figure 24). These data suggested that the molecular weight of WR4 was 210. This molecular weight would be consistent with cleavage of the two alkyl sidechains. Additional work is planned to further elucidate the structure of this metabolite.

b.   WR3 (28 min metabolite peak)

CIMS analysis of the radiolabeled metabolite of WR242511, WR3, was inconclusive and, thus, provided no structure identification.

c.   Additional methods

Using TLC as an isolation method, some separation of the radioactive components in dog urine, which were derived from [14C]WR242511, was achieved following development of Silica Gel 60 plates in acetone. One metabolite resolved on TLC was eluted from the gel and subsequently analyzed by HPLC. The results indicated that this metabolite sample contained only a single radioactive peak and several UV absorbing peaks which had shorter retention times than the radioactive peak. Thus, this method may be useful for isolating additional metabolites of WR242511 from urine. Further work along these lines is planned.

Because of the limited quantity of RBCs which were obtained during the metabolism study with [14C]WR242511 in dogs[7], our main focus to date has been to investigate the urinary metabolites of WR242511 with the intent of developing methods applicable to the isolation of the metabolite(s) in RBCs. Data obtained during our preliminary investigations with RBCs indicated that most of the radioactivity in the cells is tightly bound. Upon successive extraction of lyophilized RBCs with methylene chloride, acetone, methanol and water, 58% of the extractable radioactivity was recovered in the methanol extract and 31% in the water extract. To date, characterization of the radioactivity in these RBC extracts has not been accomplished.

Significance. The results of investigations conducted in dogs by Marino and colleagues[5], as well as studies completed in our laboratory[7], indicate a metabolite of WR242511, which is sequestered in red blood bloods, is responsible for the methemoglobinemia produced by the compound. Thus, an understanding of the rate and route of metabolism of WR242511 is critically important for the design of therapeutic strategies for the compound.

The results of our studies indicate that WR242511 is extensively metabolized in dogs; no unchanged parent compound is eliminated in urine and only traces are detectable in feces following oral administration. Although, to date, positive structure identification has not been elucidated for any metabolites of WR242511, we have obtained some information that indicates the molecule undergoes dealkylation. This reaction has been shown to occur with primaquine, a related aminoquinoline compound[31]. It will be of interest to determine whether the metabolite(s) retained in red blood cells is also eliminated in urine.

IV. CONCLUSIONS

1. Following i.m. administration to dogs, [^{14}C]HI-6 was extensively metabolized. In addition to intact HI-6, five predominant radiolabeled and one unlabeled metabolite were detected in urine. From mass spectral and chromatographic data, the major urinary metabolite was identified as pyridine-2-aldoxime. Evidence was obtained for the formation of a pyridone derivative of HI-6. Other metabolites isolated in urine included a volatile radiolabeled metabolite, picolinic acid and several minor, relatively polar metabolites.

2. In dogs administered either an oral or i.v. dose of 7 mg/kg of [^{14}C]PAHP, no unchanged PAHP was detected in urine. The majority of the radioactivity in urine was associated with three metabolite peaks. One radiolabeled metabolite peak was found to consist of two components, identified as metabolites M1 and M2. Mass spectral data suggested that M1 (mass, 237) was 6-[4'-hydroxyaminobenzoyl]hexanol and that M2 (mass, 265) was methyl 6-[4'-hydroxyaminobenzoyl]hexoate. Proposed NMR analyses should provide structure confirmation of these two metabolites. Efforts to identify the other two major urinary metabolites are in progress. In feces, unchanged PAHP was detected following either oral or i.v. administration of the compound. In addition, two major metabolites and several minor metabolites, all of which were more polar than PAHP, were detected. Chromatographic analyses indicated that the radiolabeled metabolites of PAHP eliminated in feces were different from those excreted in urine. The identity of the fecal metabolites is under investigation. The apparent formation of 4-hydroxyamino derivatives of PAHP may pose a potential toxicological threat in view of the reported toxicity of N-hydroxylated aromatic amines.

3. In rats administered either an oral or i.v. dose of [^{14}C]PAHP, no unchanged parent compound was eliminated in urine. Two major radiolabeled metabolites, and several minor radiolabeled components, were detected in urine. The two major urinary metabolites excreted by rats differed from the urinary metabolites excreted by dogs in both spectral and chromatographic properties. From mass spectral analysis of the rat urinary metabolite identified as M6, the molecular weight of the compound was determined to be 249; the metabolite was tentatively identified as 6-[4'-hydroxy-aminobenzoyl]hexanol. The identity of the other major urinary metabolite is under investigation. In feces, unchanged PAHP and two major radiolabeled metabolites were resolved. The retention time of the metabolites in rat feces was similar to that for the metabolites resolved in dog feces. Work is in progress to isolate and identify the metabolites eliminated in feces. The results may indicate that a species difference in the metabolism of PAHP exists. If so, the results derived from experiments conducted in one or both of these species may not be applicable to humans.

4. To date, the results of HPLC analyses conducted on urine collected from dogs administered [^{14}C]WR242511 indicate the compound is metabolized to at least five radiolabeled compounds. The isolation and identification of these urinary metabolites have been hampered by the low levels of the individual metabolites excreted in urine and by difficulties in developing a chromatographic system that will effect adequate retention and resolution of the metabolites. Preliminary data indicate that the molecule undergoes dealkylation. Efforts also have been directed towards identifying the metabolite(s) that becomes sequestered in RBCs following administration of [^{14}C]WR242511. The goal of these efforts is to ultimately identify the metabolite(s) responsible for the methemoglobinemia observed after administration of WR242511.

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Table 1: Summary of the Urinary Elimination of Unchanged [^{14}C]HI-6 and Metabolites following I.M. Administration of [^{14}C]HI-6 Dichloride to Dogs

Dog	Sample Time(hr)	2-4	12	14	19	22	23	32 (HI-6)
1134	0-2	0.5	0.2	0.6	7.2	2.1	ND	36.2
	6-8	2.2	0.7	1.2	5.4	4.5	1.4	13.7
	8-12	0.2	0.2	0.1	0.2	0.2	ND	1.0
	Total	2.9	1.1	1.9	12.9	6.8	1.4	50.9
1135	0-2	0.4	ND	0.3	1.5	1.1	0.2	19.3
	2-4	1.1	0.2	0.7	6.6	1.9	1.2	15.3
	4-6	1.0	0.2	0.4	2.1	2.0	0.4	8.5
	6-8	0.2	0.1	0.05	0.3	0.1	0.05	1.8
	8-12	0.2	0.1	0.1	0.3	0.3	ND	0.8
	Total	2.8	0.6	1.6	10.8	5.4	1.8	45.7
1144	8-12	11.0	2.6	5.5	18.5	13.7	4.5	28.0
	12-24	0.9	0.4	0.1	0.2	1.5	ND	0.6
	Total	11.8	3.1	5.6	18.7	15.2	4.5	28.6
1145	8-12	8.9	2.6	5.7	16.4	11.8	3.9	25.7
	12-24	1.1	0.4	0.2	0.5	1.8	0.1	1.5
	Total	10.0	3.0	6.0	17.0	13.6	4.1	27.2
1146	0-2	0.05	ND	ND	0.1	0.8	ND	2.2
	4-6	4.1	0.5	3.4	12.7	3.8	1.9	48.9
	8-12	0.5	0.5	ND	0.7	1.0	0.2	1.0
	Total	4.6	1.0	3.4	13.6	5.6	2.0	52.1
1147	0-2	0.2	ND	0.2	0.8	ND	ND	12.7
	2-4	0.2	ND	0.4	0.7	0.2	ND	9.7
	4-6	1.3	0.3	1.1	5.1	1.0	ND	11.7
	8-12	0.8	0.2	0.4	1.2	1.0	ND	2.6
	Total	2.5	0.6	2.2	7.8	2.2	0.0	36.7

[a]Values were calculated from radioassay data, except those for the 22 min peak which were calculated from the molar extinction coefficient of HI-6.

Table 2. Metabolite Profiles of Urine Collected Following Oral
Administration of 7 mg/kg of [^{14}C]PAHP to Dogs[a]

Sample Time (hr)	Dog	% Radioactivity Eluting at (min):								
		9	11	14	17	21	22	24	26[b]	28
0-6	1148	7.9	9.7	8.4	2.7	6.7	8.4	6.5	36.2	9.7
	1149	12.3	15.7	3.3	3.6	7.1	8.4	8.5	30.7	4.6
	1151	10.8	12.8	6.4	2.7	5.3	6.1	4.6	41.1	ND[c]
	1152	16.1	14.8	2.8	8.8	4.1	3.7	5.7	28.0	2.9
	1153	14.1	15.7	6.8	5.5	3.6	4.7	6.2	32.2	3.6
Mean		12.2	13.7	5.6	4.7	5.4	6.3	6.3	33.6	4.1
6-12	1148	7.9	11.5	4.7	3.5	8.0	5.7	8.3	37.3	7.7
	1149	10.1	23.0	10.4	5.7	7.1	4.0	4.3	21.5	3.9
	1151	10.0	24.6	7.8	5.1	3.6	3.1	3.7	24.9	6.9
	1152	10.2	29.0	1.3	8.4	4.9	2.9	4.9	22.9	5.0
	1153	12.3	24.7	ND	9.7	4.4	ND	3.2	34.9	4.4
Mean		10.1	22.6	4.8	6.5	5.6	3.1	4.9	28.3	5.6
12-24	1148	6.8	13.7	10.0	6.6	9.1	3.1	5.6	32.6	6.0
	1149	9.5	20.8	11.7	5.7	12.3	ND	2.6	24.4	3.3
	1151	9.7	23.4	7.4	3.6	5.6	ND	2.0	37.3	ND
	1152	12.7	24.4	13.9	6.0	11.8	ND	ND	31.2	ND
Mean		9.7	20.6	10.8	5.5	9.7	0.8	2.5	31.4	2.3

[a]The retention time of unchanged PAHP was 48 min.
[b]Peak consisted of two metabolites, M1 and M2, which were not separated under the HPLC conditions of analysis used.
[c]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 3. Urinary Elimination of Metabolites Following Oral
Administration of 7 mg/kg of [^{14}C]PAHP to Dogs

Sample Time (hr)	Dog	\% of Dose/Metabolite Eluting at (min):								
		9	11	14	17	21	22	24	26[a]	28
0-6	1148	3.0	3.8	3.3	1.0	2.6	3.3	2.5	14.0	3.8
	1149	4.1	5.2	1.1	1.2	2.3	2.8	2.8	10.1	1.5
	1151	2.0	2.3	1.2	0.5	1.0	1.1	0.8	7.5	ND[b]
	1152	4.3	4.0	0.8	2.4	1.1	1.0	1.5	7.5	0.8
	1153	4.6	5.1	2.2	1.8	1.2	1.6	2.0	10.6	1.2
Mean		3.6	4.1	1.7	1.4	1.6	1.9	1.9	9.9	1.4
6-12	1148	1.5	2.1	0.9	0.7	1.5	1.1	1.6	7.0	1.4
	1149	1.6	3.7	1.7	0.9	1.1	0.6	0.7	3.5	0.6
	1151	1.8	4.5	1.4	0.9	0.7	0.6	0.7	4.5	1.2
	1152	1.5	4.4	0.2	1.3	0.7	0.4	0.7	3.5	0.8
	1153	2.0	4.1	ND	1.6	0.7	ND	0.5	5.8	0.7
Mean		1.7	3.8	0.8	1.1	1.0	0.5	0.8	4.8	1.0
12-24	1148	0.5	0.9	0.7	0.4	0.6	0.2	0.4	2.2	0.4
	1149	0.7	1.6	0.9	0.4	1.0	ND	0.2	1.9	0.3
	1151	1.7	4.1	1.3	0.6	1.0	ND	0.4	6.6	ND
	1152	1.1	2.1	1.2	0.5	1.0	ND	ND	2.6	ND
Mean		1.0	2.2	1.0	0.5	0.9	0.1	0.2	0.2	

[a]Peak consisted of two metabolites, M1 and M2, which were not separated under the HPLC
conditions of analysis used.

[b]ND = not detectable; radioactivity was not detected at the indicated retention time.

Table 4. Metabolite Profiles of Urine Collected Following I.V.
Administration of 7 mg/kg of [^{14}C]PAHP to Dogs[a]

Sample Time (hr)	Dog	% Radioactivity Eluting at (min):								
		9	11	14	17	21	22	24	26[b]	28
0-6	1148	13.0	12.6	9.2	5.7	4.5	6.1	6.7	28.3	7.5
	1149	11.8	13.6	4.1	10.1	6.5	5.1	5.6	28.7	5.2
	1151	16.3	19.8	4.0	3.2	6.2	4.3	5.4	31.1	7.4
	1152	14.7	17.3	5.2	9.4	4.5	5.2	6.1	20.1	3.3
Mean		11.3	17.1	8.0	8.4	5.0	4.5	5.6	23.6	3.2
6-12	1148	8.1	17.0	11.0	7.5	5.7	3.8	5.1	27.4	ND[c]
	1149	10.9	26.7	2.2	9.6	5.4	2.2	5.2	23.3	5.4
	1151	13.0	27.6	8.0	6.5	6.6	2.7	1.3	21.8	7.2
	1152	11.2	20.1	8.9	8.7	5.0	2.7	4.8	22.6	5.7
	1153	13.3	15.9	6.0	5.6	4.6	4.1	5.4	26.6	8.7
Mean		11.3	21.5	7.2	7.6	5.4	3.1	4.4	24.3	6.7
12-24	1148	8.1	20.0	ND	6.5	9.9	ND	4.7	29.5	3.5
	1149	12.4	24.4	12.3	3.2	9.9	ND	5.3	23.4	4.1
	1151	8.6	27.1	ND	4.2	6.4	2.8	1.5	34.1	4.7
	1152	13.2	20.8	11.7	5.3	8.3	ND	1.2	27.4	ND
Mean		10.6	23.1	6.0	4.8	8.6	0.7	3.2	28.6	3.1

[a]The retention time of unchanged PAHP was 48 min.
[b]Peak consisted of two metabolites, M1 and M2, which were not separated under the HPLC conditions of analyses used.
[c]ND , not detectable; radioactivity was not detected at the indicated retention time.

Table 5. Urinary Elimination of Metabolites Following I.V. Administration of 7 mg/kg of [^{14}C]PAHP to Dogs

Sample Time (hr)	Dog	% of Dose/Metabolite Eluting at (min): 9	11	14	17	21	22	24	26[a]	28
0-6	1148	5.0	4.8	3.5	2.2	1.7	2.3	2.6	10.9	2.9
	1149	3.6	4.1	1.2	3.1	2.0	1.6	1.7	8.7	1.6
	1151	4.8	5.8	1.2	0.9	1.8	1.2	1.6	9.1	2.2
	1152	6.1	7.2	2.1	3.9	1.8	2.1	2.5	8.3	1.4
Mean		4.8	5.5	2.0	2.5	1.8	1.8	2.1	9.2	2.0
6-12	1148	0.3	0.7	0.4	0.3	0.2	0.1	0.2	1.0	ND
	1149	1.0	2.5	0.2	0.9	0.5	0.2	0.5	2.2	0.5
	1151	0.9	1.8	0.5	0.4	0.4	0.2	0.1	1.4	0.5
	1152	0.5	1.0	0.4	0.4	0.2	0.1	0.2	1.1	0.3
	1153	6.5	7.8	2.9	2.7	2.2	2.0	2.6	13.0	4.3
Mean		1.8	2.7	0.9	1.0	0.7	0.5	0.7	3.8	1.1
12-24	1148	0.2	0.5	ND	0.2	0.3	ND	0.1	0.8	0.1
	1149	0.9	1.8	0.9	0.2	0.7	ND	0.4	1.7	0.3
	1151	1.3	4.2	ND	0.7	1.0	0.4	0.2	5.3	0.7
	1152	1.4	2.2	1.2	0.6	0.9	ND	0.1	2.9	ND
Mean		1.0	2.2	0.5	0.4	0.7	0.1	0.2	2.7	0.3

[a]Peak consisted of two metabolites, M1 and M2, which were not separated under the conditions of analysis used.

Table 6. Metabolite Profiles of Urine Collected Following Oral
Administration of 7 mg/kg of [^{14}C]PAHP to Rats[a]

Sample Time (hr)	Rat	% Radioactivity Eluting at (min)				
		21	22	24[b]	25	26
0-6	94	24.7	7.4	1.4	5.1	54.3
	95	16.1	8.6	7.0	6.2	47.6
	96	24.0	8.3	4.7	7.3	38.8
	97	21.3	8.6	4.2	3.4	46.5
	98	18.7	7.6	6.3	5.8	47.4
	99	19.1	8.9	6.3	5.0	45.0
Mean		20.7	8.2	5.0	5.5	46.6
6-12	94	18.0	11.1	9.8	1.6	35.7
	95	14.7	10.2	8.3	1.8	43.9
	96	12.8	12.7	6.5	2.4	32.5
	97	17.0	11.8	9.5	1.2	37.4
	98	16.1	12.0	8.5	1.9	38.4
	99	17.0	10.0	8.8	2.8	37.6
Mean		15.9	11.3	8.6	1.9	37.6

[a]The retention time of unchanged PAHP was 48 min.
[b]Two radioactive peaks.

Table 7.   Urinary Elimination of Metabolites Following Oral
Administration of 7 mg/kg of [^{14}C]PAHP to Rats

| Sample Time (hr) | Rat | % of Dose/Metabolite Eluting at (min): | | | | |
		21	22	24[a]	25	26
0-6	94	14.8	4.4	0.5	3.1	32.6
	95	6.2	3.3	2.7	2.4	18.3
	96	14.3	4.9	1.1	4.3	23.0
	97	12.7	5.1	1.3	2.0	27.7
	98	12.3	5.0	2.1	3.8	31.1
	99	10.5	4.9	2.1	2.7	24.8
Mean		11.8	4.6	1.6	3.1	26.3
6-12	94	1.8	1.1	0.6	0.2	3.6
	95	1.9	1.3	0.7	0.2	5.7
	96	1.2	1.2	0.2	0.2	3.0
	97	1.8	1.3	0.7	0.1	4.1
	98	1.2	0.9	0.4	0.1	2.9
	99	2.3	1.3	0.8	0.4	5.1
Mean		1.7	1.2	0.6	0.2	4.1

[a]Two radioactive peaks.

Table 8. Metabolite Profiles of Urine Collected Following I.V.
Administration of 7 mg/kg of [^{14}C]PAHP to Rats[a]

Sample Time (hr)	Rat	% Radioactivity Eluting at (min):				
		21	22	24[b]	25	26
0-6	145	23.3	10.9	5.0	1.8	36.6
	146	22.7	7.0	4.2	1.9	52.0
	147	28.9	12.3	3.8	0.8	45.0
	148	25.1	12.7	2.9	ND	48.9
	149	23.3	7.9	5.4	1.9	54.4
	150	26.1	8.5	4.6	1.9	41.6
Mean		24.9	9.9	4.3	1.4	46.4
6-12	145	21.2	14.8	8.4	1.6	31.3
	146	16.8	12.8	9.9	1.7	37.4
	147	23.2	12.6	10.4	3.1	36.8
	148	27.0	14.6	9.3	0.9	39.6
	149	20.8	13.5	12.7	2.8	39.5
	150	21.8	9.1	7.5	1.5	34.1
Mean		21.8	12.9	9.7	1.9	36.4

[a]The retention time of unchanged PAHP was 48 min.
[b]Two radioactive peaks.

Table 9. Urinary Elimination of Metabolites Following I.V.
Administration of 7 mg/kg of [^{14}C]PAHP to Rats

Sample Time (hr)	Rat	% of Dose/Metabolite Eluting at (min):				
		21	22	24[a]	25	26
0-6	145	16.2	7.6	2.0	1.2	25.5
	146	14.5	4.5	1.9	1.2	33.2
	147	20.0	8.5	1.4	0.5	31.1
	148	16.6	8.4	1.9	ND	32.3
	149	13.3	4.5	2.1	1.1	31.1
	150	17.2	5.6	3.0	1.2	27.4
Mean		16.3	6.5	2.1	1.0	30.1
6-12	145	0.9	0.6	0.2	0.1	1.3
	146	2.5	1.9	0.7	0.2	5.5
	147	2.9	1.6	1.0	0.4	4.7
	148	4.0	2.1	1.0	0.1	5.8
	149	1.2	0.8	0.5	0.2	2.2
	150	1.5	1.1	0.3	0.2	3.9
Mean		2.2	1.3	0.6	0.2	3.9

[a]Two radioactive peaks.

Table 10. Metabolite Profiles of Feces Collected Following
Oral Administration of 7 mg/kg of [^{14}C]PAHP to Dogs[a]

Sample Time (hr)	Dog	18	23	25-26	28	30	35	39	42	48
		\% Radioactivity Eluting at (min):								
0-6	1149	ND	ND[b]	5.9	ND	ND	4.2	3.9	10.4	67.1
	1151	2.6	ND	13.2	2.9	1.5	1.2	1.9	16.4	56.8
	1152	ND	ND	17.6	5.3	ND	ND	3.7	13.7	56.6
	1153	3.1	ND	18.3	3.0	0.6	1.4	3.3	9.0	55.3
Mean		1.4	ND	13.8	2.8	0.5	1.7	3.2	12.4	58.9
12-24	1148	4.0	6.8	29.4	6.8	5.1	4.1	4.4	11.7	24.9
	1149	7.1	ND	21.4	4.8	ND	3.0	3.5	18.4	38.2
	1151	3.6	6.7	29.5	4.0	5.6	ND	2.9	30.2	12.1
	1152	3.5	2.2	15.5	3.3	ND	ND	ND	14.6	55.6
Mean		4.5	3.9	23.9	4.7	2.7	1.8	2.7	18.7	32.7

[a]The retention time of unchanged PAHP was 48 min.

[b]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 11. Fecal Elimination of Metabolites Following Oral Administration of 7 mg/kg of [^{14}C]PAHP to Rats

Sample Time (hr)	Rat	_18	_21	24-25	_26	_29	_42	_48
				% of Dose/Metabolite Eluting at (min):				
6-12	1148	0.04	ND[a]	0.2	ND	ND	ND	0.1
	1149	ND	ND	0.3	ND	ND	0.2	0.2
	1151	0.1	ND	0.4	0.1	0.05	0.04	0.1
	1152	ND	ND	0.5	0.2	ND	ND	0.1
	1153	0.6	ND	3.4	0.6	0.1	0.3	0.6
Mean		0.1	ND	1.0	0.2	< 0.05	0.1	0.2
12-24	1148	0.6	1.0	4.2	1.0	0.7	0.6	0.6
	1149	1.4	ND	4.1	0.9	ND	0.6	0.7
	1151	0.7	1.3	5.8	0.8	1.1	ND	0.6
	1152	0.6	0.4	2.8	0.6	ND	ND	ND
Mean		0.8	0.7	4.2	0.8	0.5	0.3	0.5

[a]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 12. Metabolite Profiles of Feces Collected Following I.V. Administration of 7 mg/kg of [^{14}C]PAHP to Dogs[a]

Sample Time (hr)	Dog	__ % Radioactivity Eluting at (min): __								
		18	23	25-26	28	30	35	39	42	48
6-12	1148	3.4	ND[b]	32.2	8.6	3.3	2.3	2.2	16.8	24.5
	1152	5.3	ND	30.3	14.3	ND	ND	2.8	18.3	27.1
Mean		4.4	ND	31.2	11.4	1.6	1.2	2.5	17.5	25.8
12-24	1148	5.1	16.7	35.0	10.5	3.4	ND	ND	13.1	16.3
	1151	5.0	2.0	32.1	4.3	12.6	ND	ND	27.1	13.7
	1153	3.9	ND	27.0	4.9	3.5	2.7	4.0	23.7	25.9
Mean		4.7	6.2	31.4	6.6	6.5	0.9	1.3	21.3	18.6
24-48	1151	4.0	ND	28.4	ND	18.4	7.0	3.4	26.1	7.6

[a]The retention time of unchanged PAHP was 48 min.

[b]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 13. Fecal Elimination of Metabolites Following
I.V. Administration of 7 mg/kg of [^{14}C]PAHP to Dogs[a]

Sample Time (hr)	Dog	% of Dose/Metabolite Eluting at (min):								
		18	23	25-26	28	30	35	39	42	48
6-12	1148	0.2	ND[b]	2.0	0.5	0.2	0.1	0.1	1.0	1.5
	1152	0.2	ND	1.2	0.6	ND	ND	0.1	0.7	1.1
Mean		0.2	ND	1.6	0.5	0.1	0.1	0.1	0.9	1.3
12-24	1148	0.7	2.2	4.7	1.4	0.4	ND	ND	1.7	2.2
	1151	0.5	0.2	3.2	0.4	1.3	ND	ND	2.7	1.4
	1153	0.6	ND	3.8	0.7	0.5	0.4	0.6	3.3	3.7
Mean		0.6	0.8	3.9	0.8	0.7	0.1	0.2	2.6	2.4
24-48	1151	ND	ND	1.1	0.1	0.9	0.3	0.1	1.1	0.4

[a]The retention time of unchanged PAHP was 48 min.

[b]ND, not dectable; radioactivity was not detected at the indicated retention time.

Table 14. Metabolite Profiles of Feces Collected Following Oral
Administration of 7 mg/kg of [^{14}C]PAHP to Rats[a]

Sample Time (hr)	Rat	% Radioactivity Eluting at (min):						
		18	21	24-25	26	29	42	48
6-12	95	ND[b]	ND	ND	18.4	15.1	21.6	44.9
	96	8.2	8.2	13.0	24.5	15.9	10.0	20.4
	97	ND	11.5	ND	28.1	28.4	15.9	16.3
	98	ND	ND	20.3	44.5	35.3	ND	ND
Mean		2.1	4.9	8.3	28.9	23.7	11.9	20.4
12-24	94	ND	8.7	12.0	40.1	16.3	10.7	12.1
	95	ND	9.1	9.,2	24.9	19.2	10.1	27.5
	96	7.2	15.4	18.2	28.7	16.3	4.7	2.6
	97	ND	12.7	13.5	32.7	19.2	10.5	5.7
	98	ND	13.1	6.1	40.3	33.2	ND	ND
	99	ND	10.5	14.4	27.2	18.9	10.9	10.5
Mean		1.2	11.6	12.2	32.3	20.5	7.8	9.7

[a]The retention time of unchanged PAHP was 48 min.
[b]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 15. Fecal Elimination of Metabolites Following Oral Administration of 7 mg/kg of [^{14}C]PAHP to Rats

Sample Time (hr)	Rat	% of Dose/Metabolite Eluting at (min):						
		18	21	24-25	26	29	42	48
6-12	95	ND[a]	ND	ND	0.6	0.5	0.7	1.5
	96	0.4	0.4	0.6	1.1	0.7	0.5	0.9
	97	ND	0.5	ND	1.3	1.3	0.8	0.8
	98	ND	ND	0.9	2.0	1.6	ND	ND
Mean		0.1	0.5	0.2	1.3	1.0	0.6	1.1
12-24	94	ND	1.3	1.8	6.0	2.4	1.6	1.8
	95	ND	2.7	2.7	7.3	5.7	3.0	8.1
	96	0.7	1.6	1.9	2.9	1.7	0.5	0.3
	97	ND	1.7	1.8	4.4	2.6	1.4	0.8
	98	ND	1.0	0.5	3.2	2.6	ND	ND
	99	ND	1.4	1.9	3.6	2.5	1.4	1.4
Mean		0.1	1.6	1.8	4.6	2.9	1.6	2.5

[a]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 16. Metabolite Profiles of Feces Collected Following I.V. Administration of 7 mg/kg of [^{14}C]PAHP to Rats[a]

Sample Time (hr)	Rat	% Radioactivity Eluting at (min)						
		18	21	24-25	26	29	42	48
6-12	145	6.5	10.4	13.5	26.0	23.8	6.3	6.6
	149	6.3	8.6	12.2	20.4	17.4	12.1	12.1
	150	9.0	6.1	13.0	20.9	13.1	9.7	10.5
Mean		7.3	8.4	12.9	22.4	18.1	9.4	9.7
12-24	145	ND[b]	8.0	15.7	23.3	23.5	7.8	ND
	146	5.4	11.9	13.0	27.4	18.1	10.9	13.3
	147	8.1	11.5	13.8	18.7	21.2	7.0	5.2
	148	7.5	11.9	11.3	16.3	28.2	8.2	5.8
	149	8.0	7.5	17.6	26.7	23.8	9.5	ND
	150	9.8	5.1	17.9	26.9	13.5	5.5	6.6
Mean		6.5	9.3	14.9	23.2	21.4	8.2	5.2

[a]The retention time of unchanged PAHP was 48 min.

[b]ND, not detectable; radioactivity was not detected at the indicated retention time.

Table 17. Fecal Elimination of Metabolites Following I.V.
Administration of 7 mg/kg of [^{14}C]PAHP to Rats

Sample Time (hr)	Rat	\% of Dose/Metabolite Eluting at (min):						
		18	21	24-25	26	29	42	48
6-12	145	0.5	0.7	0.9	1.8	1.6	0.4	0.5
	149	0.7	0.9	1.3	2.2	1.9	1.3	1.3
	150	0.7	0.5	1.0	1.6	1.0	0.7	0.8
Mean		0.6	0.7	1.1	1.9	1.5	0.8	0.9
12-24	145	ND[a]	0.4	0.9	1.3	1.3	0.4	ND
	146	0.7	1.5	1.6	3.4	2.2	1.3	1.6
	147	0.8	1.1	1.4	1.8	2.1	0.7	0.5
	148	0.6	0.9	0.9	1.3	2.2	0.6	0.5
	149	0.2	0.2	0.5	0.8	0.7	0.3	ND
	150	0.6	0.3	1.1	1.6	0.8	0.3	0.4
Mean		0.5	0.7	1.0	1.7	1.5	0.6	0.7

[a]ND, not detectable; radioactivity was not detected at the indicated retention time.
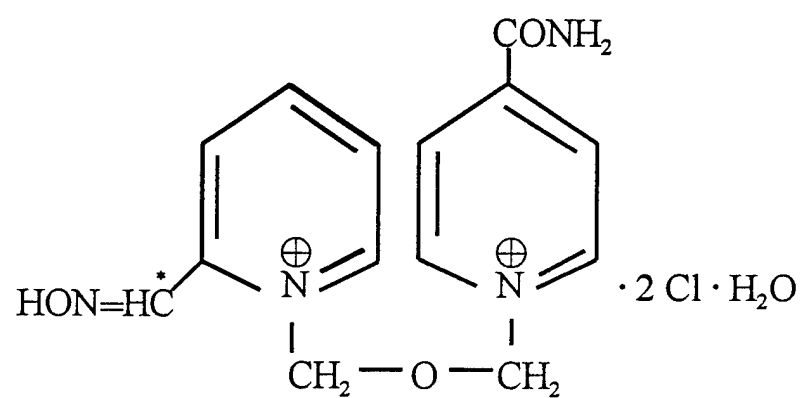
Figure 1. Structure of HI-6. The asterisk indicates the position of the radiolabel.
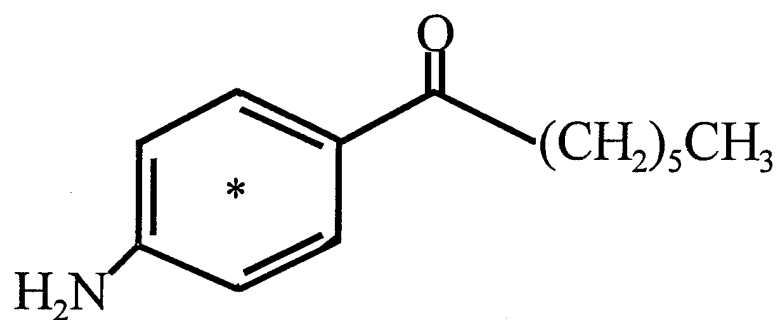
Figure 2.   Structure of PAHP. The asterisk indicates the position of the radiolabel.
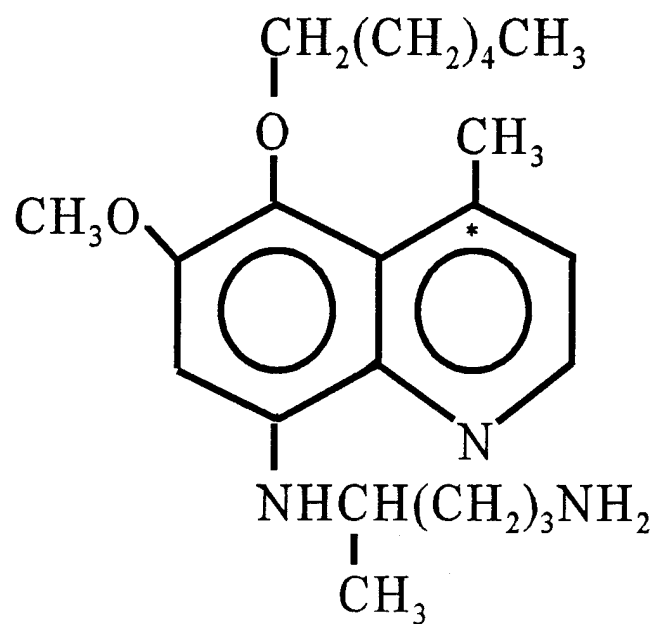
Figure 3.   Structure of WR242511. The asterisk indicates the position of the radiolabel.
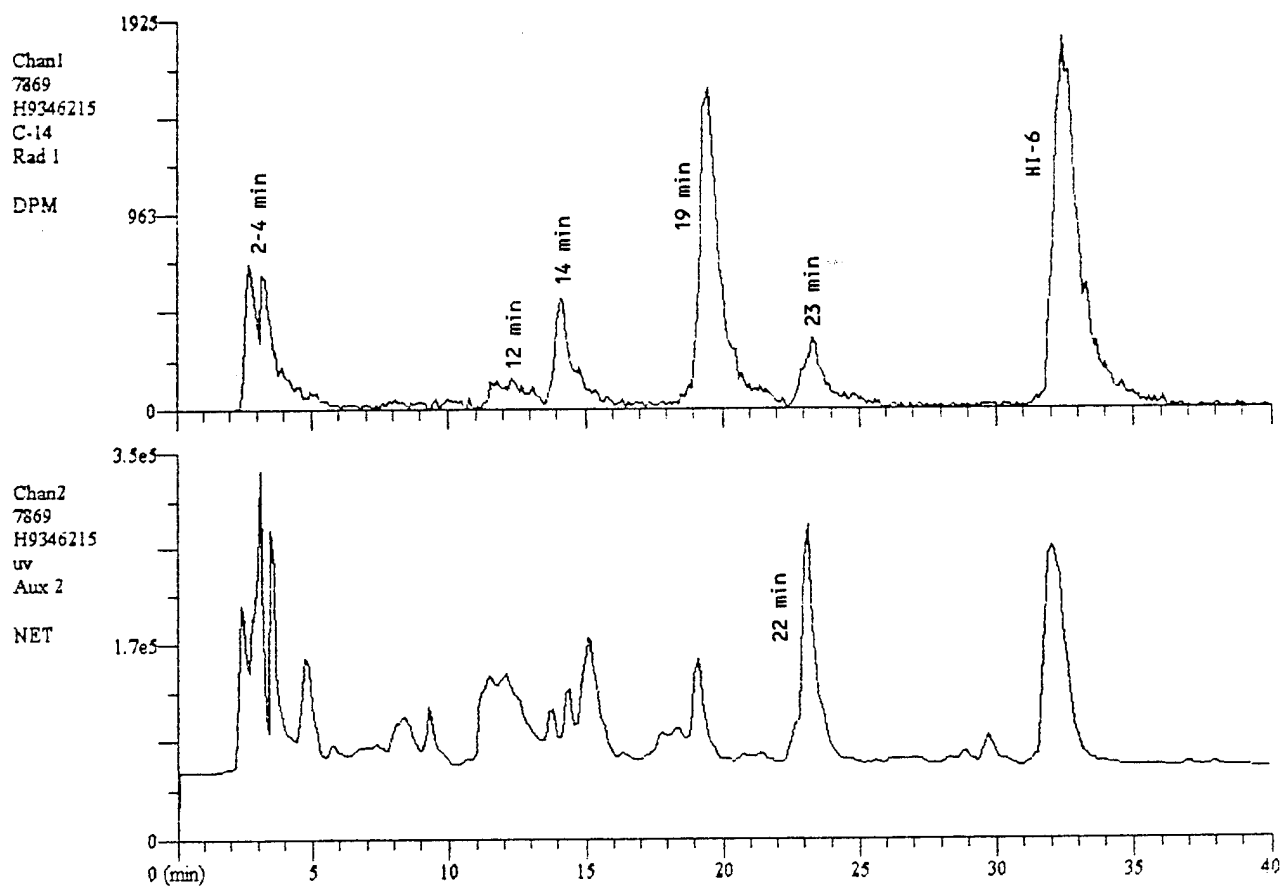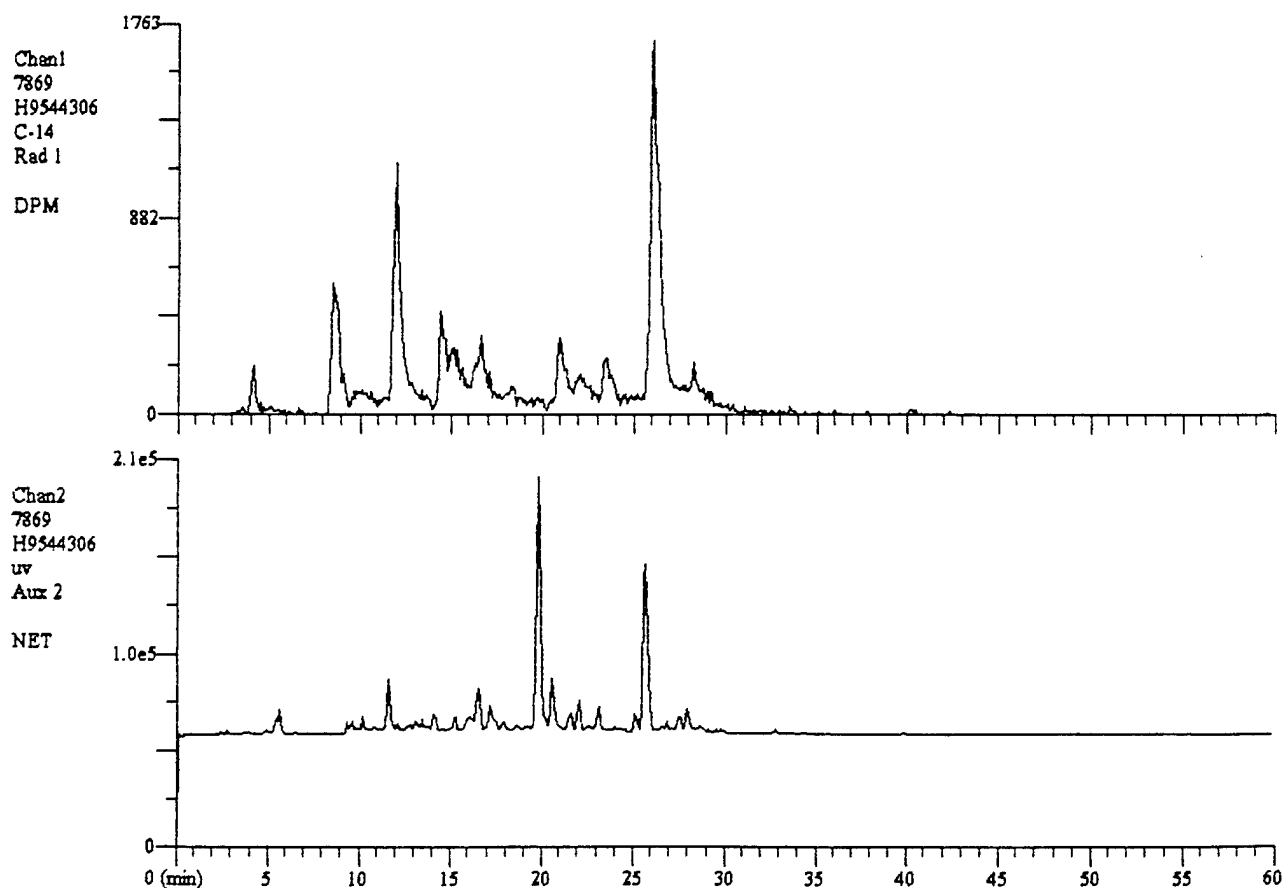
Figure 4. Representative chromatogram obtained during the HPLC analysis of urine collected from a dog administered an i.m. dose of 30 mg/kg of [^{14}C]HI-6. The upper panel shows the radioactivity profile of the sample; the lower panel shows UV absorbance at 260 nm.

SCAN GRAPHICS

Move marker to desired wavelength then release to NORMALIZE

1 H9344000 ——— 9.72- 9.05 Authentic pyridine-2-aldoxime
2 H9344001 ——— 9.05- 9.48 19 min metabolite

285   *Normalized Scans*

210.000   nm   390.000

210.000 nm 400.000

Correlation Coeff

1, 2 0.999988
1, 3
2, 3
1, 4
2, 4
3, 4
Wavelength: 284

ZOOM IN   SHIFT   TRACE   NORMALIZE   RATIO
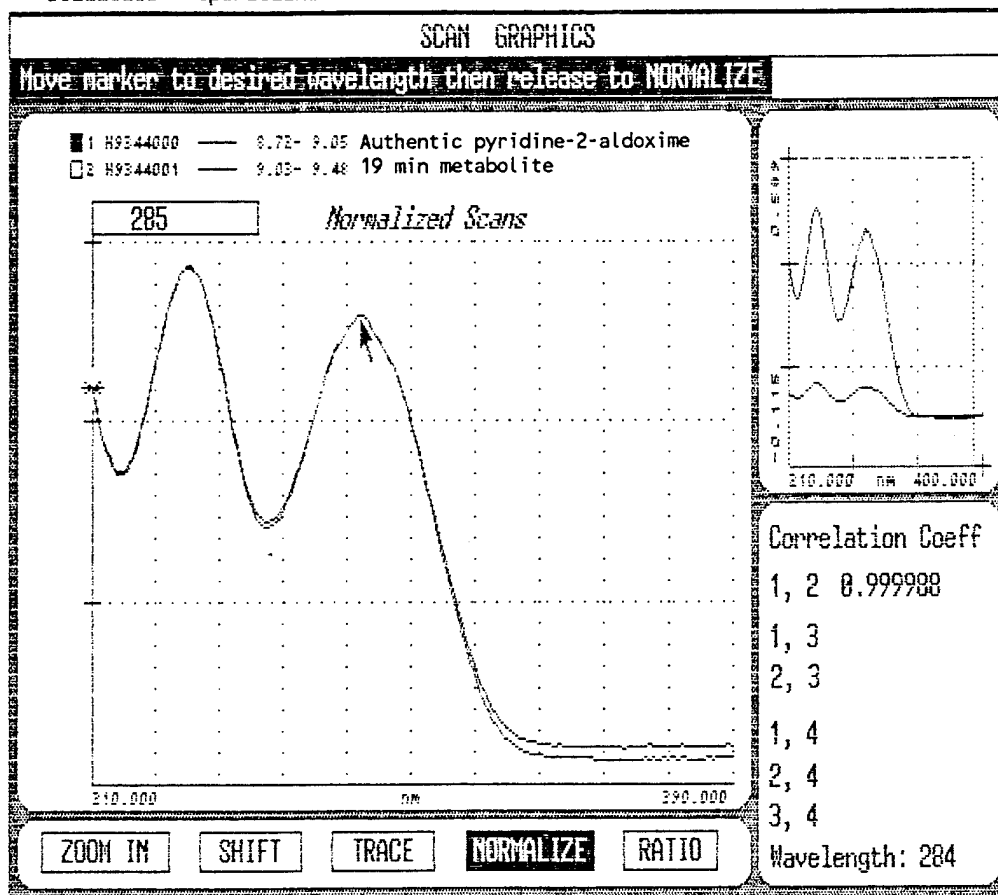
Figure 5.   UV absorbance spectra of authentic pyridine-2-aldoxime and the 19 min
metabolite of HI-6. The correlation coefficient of 0.9999 indicated that the
spectra were a good match for each other.

DI	100
ISV	5500
IN	650
OR	70
RO	30
M1	10
RE1	124.0
DM1	0.160
R1	27
L7	-100
R2	-50
M3	10
RE3	118.5
DM3	0.100
R3	-50
L9	-100
FP	-100
MU	-3800
CC	10
CG	Off
DIV	22.2
CGT	0
ISC	5622.9

+Profile Q1SCAN
Scans 937-961 minus 656-917 Time=8.33 min
Pyridine aldoxime L - 9/23/94 - 12:11 PM
Pyridine aldoxime
3 peaks
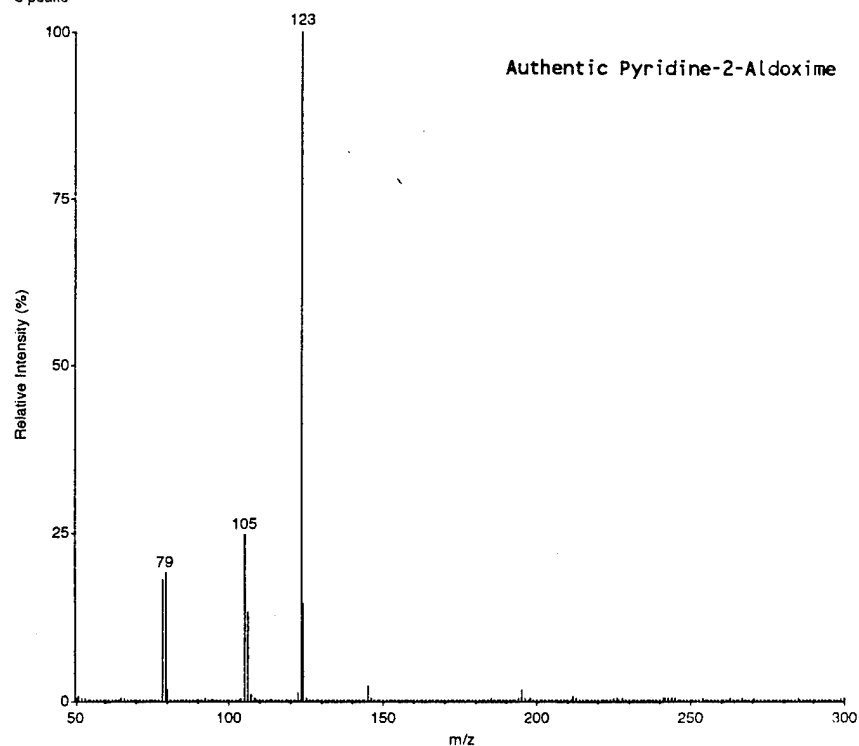
Authentic Pyridine-2-Aldoxime

DI	100
ISV	5500
IN	650
OR	70
RO	30
M1	10
RE1	124.0
DM1	0.160
R1	27
L7	-100
R2	-50
M3	10
RE3	118.5
DM3	0.100
R3	-50
L9	-100
FP	-100
MU	-3800
CC	10
CG	Off
DIV	23.06
CGT	-1.5
ISC	5618.0

+Profile Q1SCAN
Scans 907-927 minus 682-905 Time=8.00 min
H934-79   LC - 9/23/94 - 12:58 PM
H934-79
3 peaks
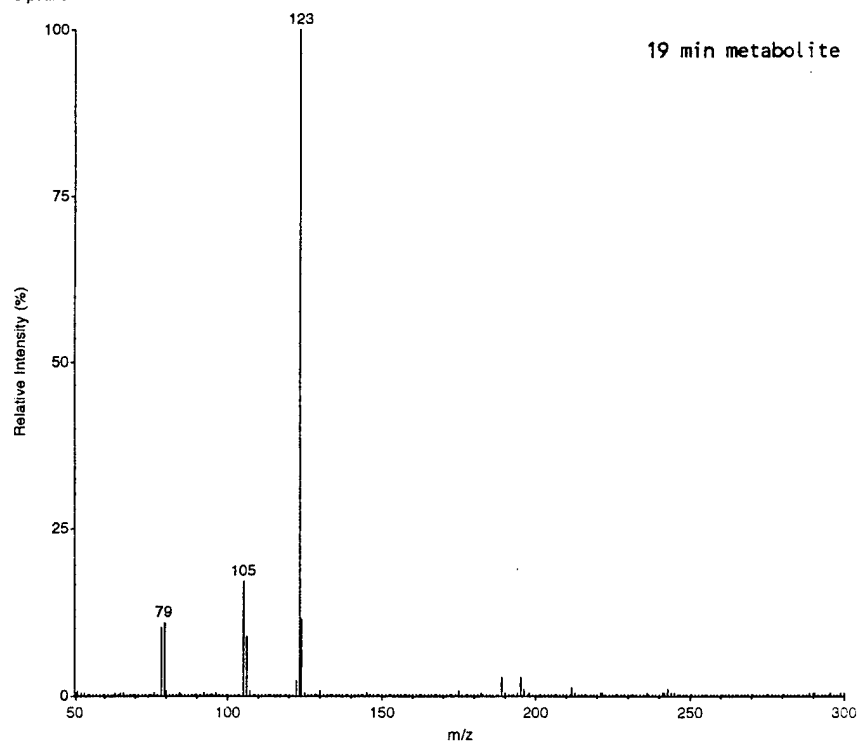
19 min metabolite

Figure 6.   Chemical ionization mass spectrum of authentic pyridine-2-aldoxime and the 19 min urinary metabolite of HI-6.
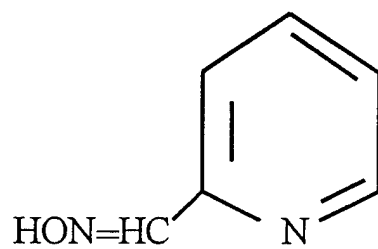
19 min Metabolite
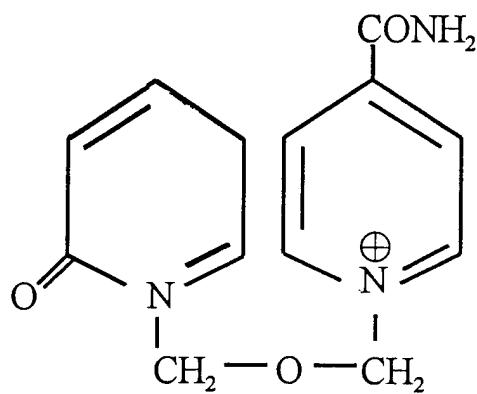
22 min Metabolite

Figure 7. Proposed structure of the 19 min and 22 min urinary metabolites of HI-6.
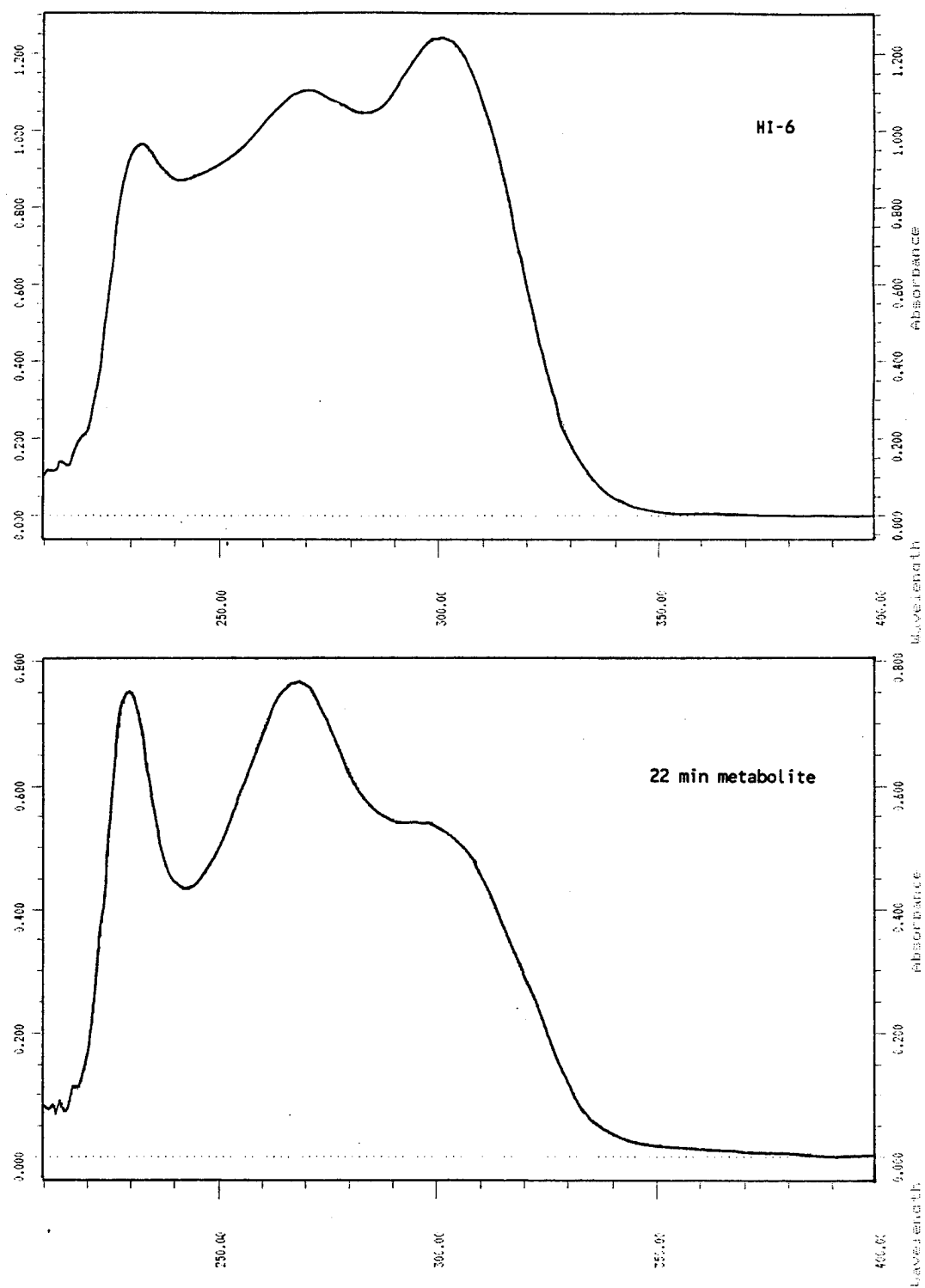
Figure 8.   UV absorbance spectra of the 22 min urinary metabolite of HI-6 and authentic HI-6.

DI 100
ISV 5500
IN 650
OR 70
RO 30
M1 10
RE1 124.0
DM1 0.160
R1 27
L7 -100
R2 -50
M3 10
RE3 118.5
DM3 0.100
R3 -50
L9 -100
FP -100
MU -3800
CC 10
CG Off
DIV 1.6
CGT 6.1
ISC 5613.09

+Profile Q1SCAN
Scans 71-83 minus 28-68 Time=1.46 min
H934-79 22 min. pea - 9/15/94 - 10:17 AM
H934-79 22 min. peak
11 peaks

4,114,709

Relative Intensity (%)

260
108
138
130
80
102
214
230
200
430 454

m/z
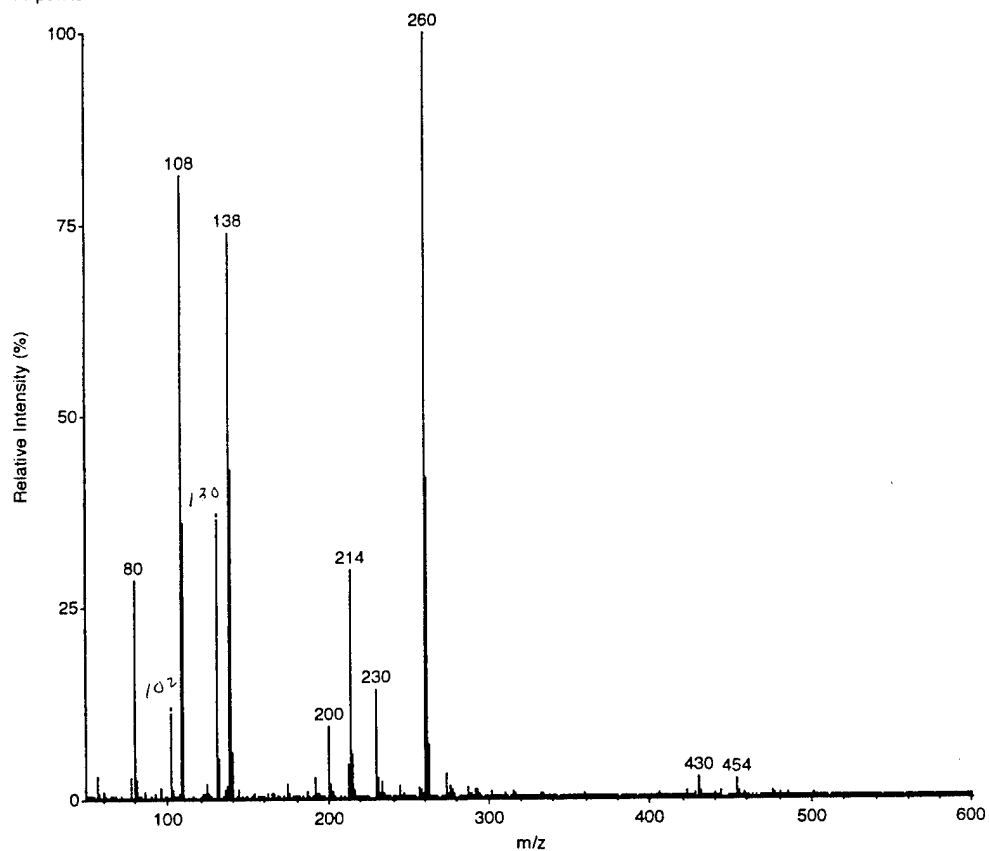
Figure 9.   Chemical ionization mass spectrum of the 22 min urinary metabolite of HI-6.
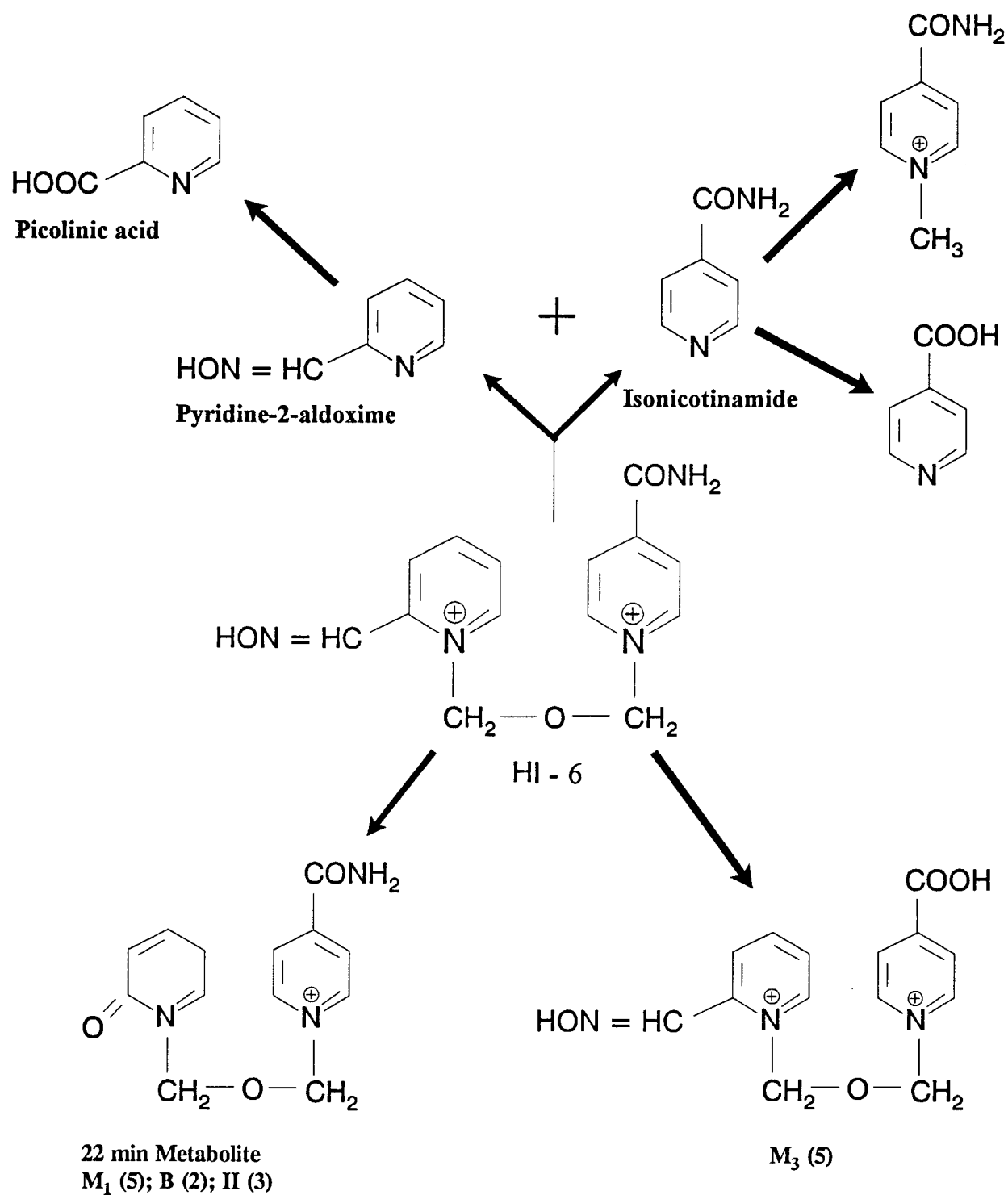
Figure 10.   Proposed scheme for the metabolism of HI-6 in dogs.

Figure 11.   Representative chromatogram obtained during the HPLC analysis of urine collected from a dog administered an i.v. dose of 7 mg/kg of [^{14}C]PAHP. The upper panel shows the radioactivity profile of the sample; the lower panel shows UV absorbance at 316 nm. In this system, PAHP eluted at 48 min.
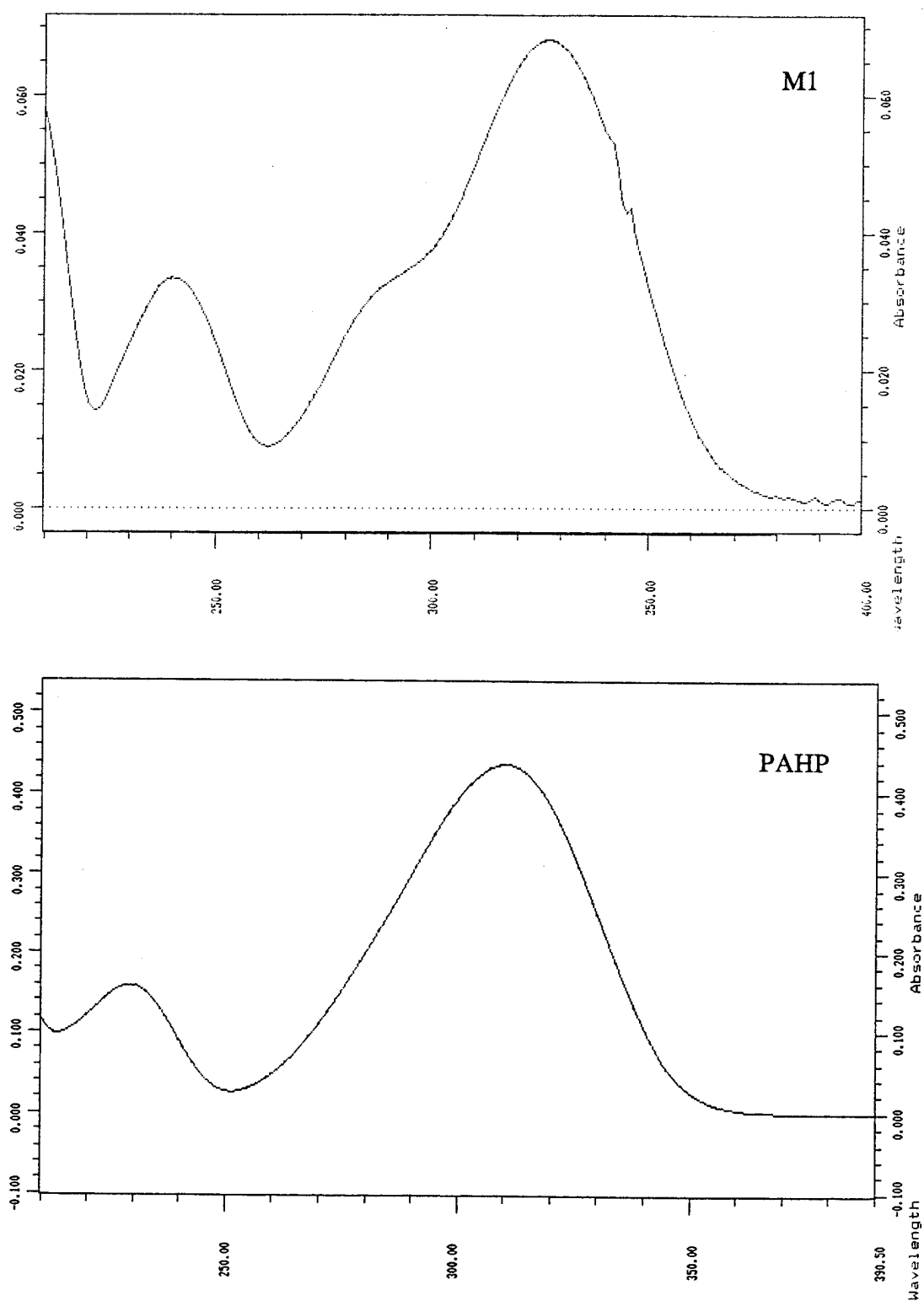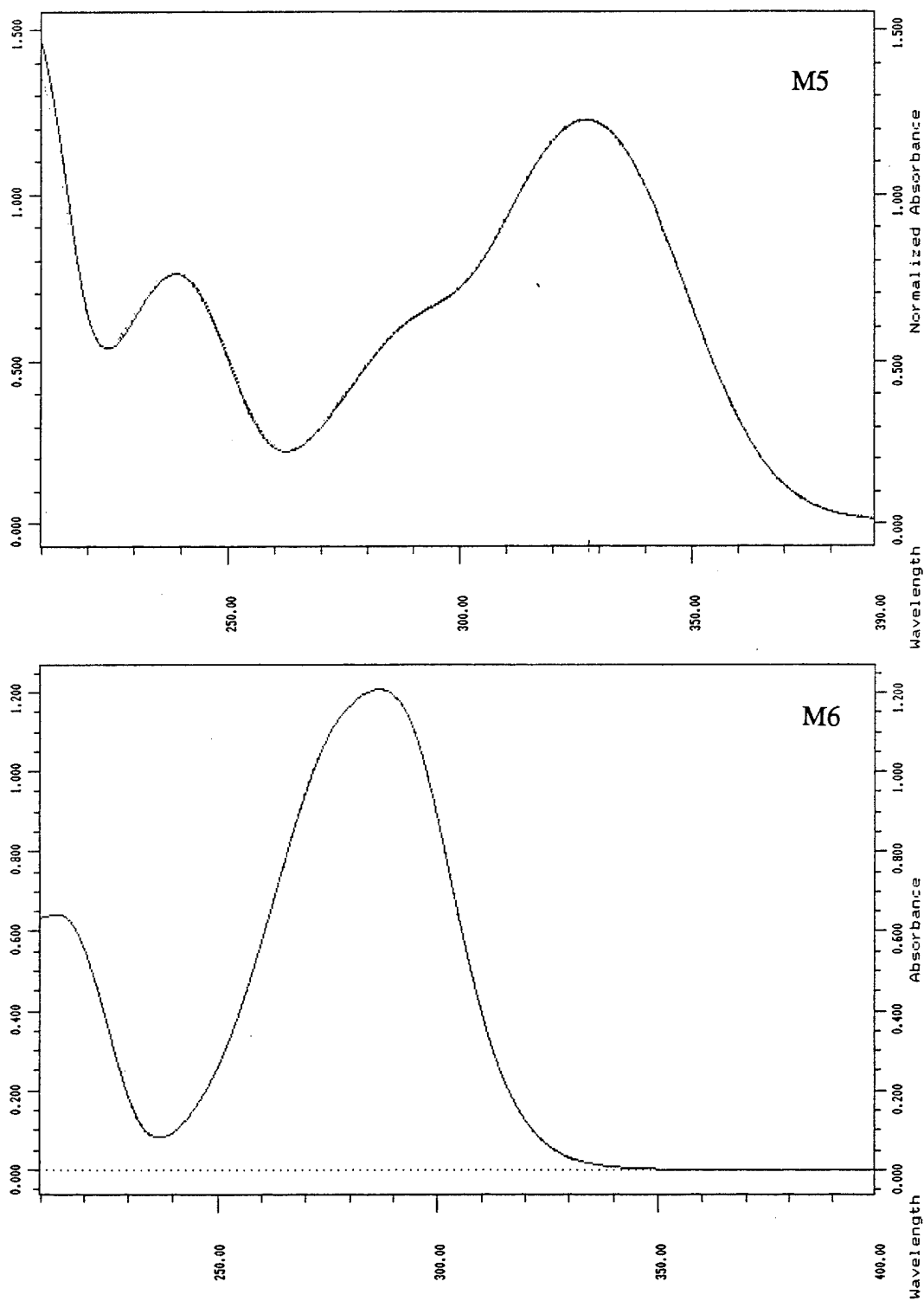
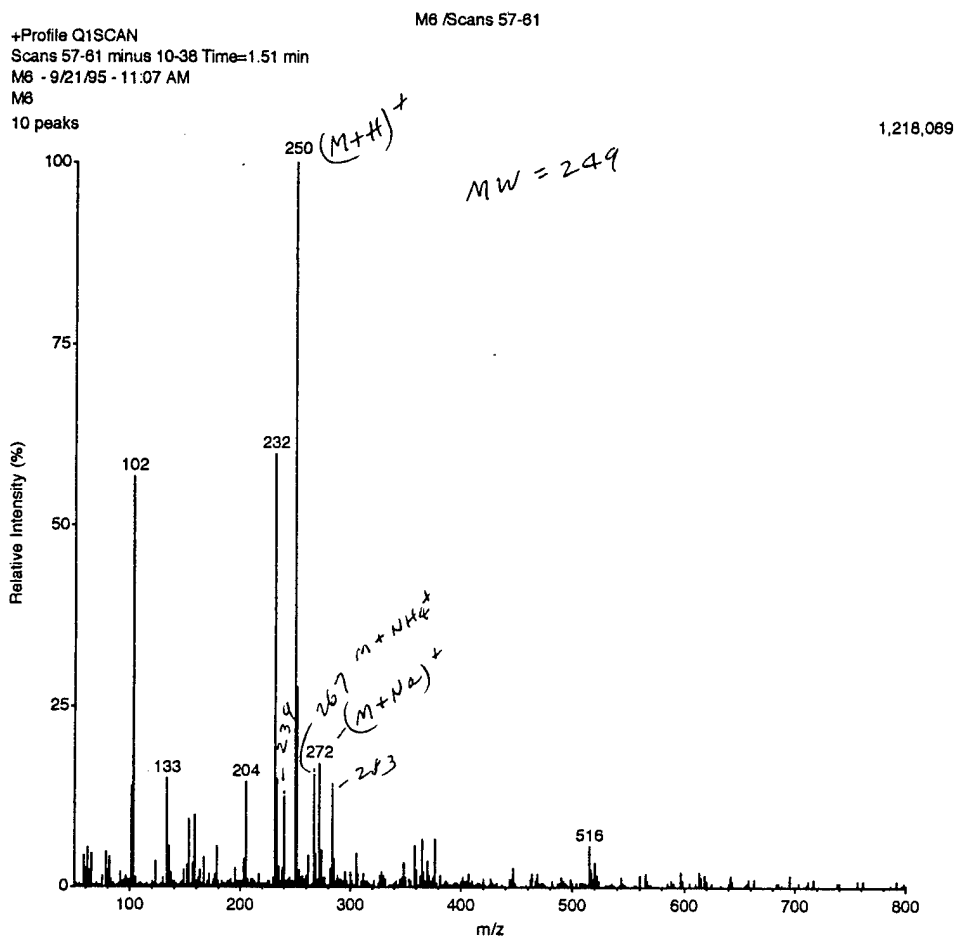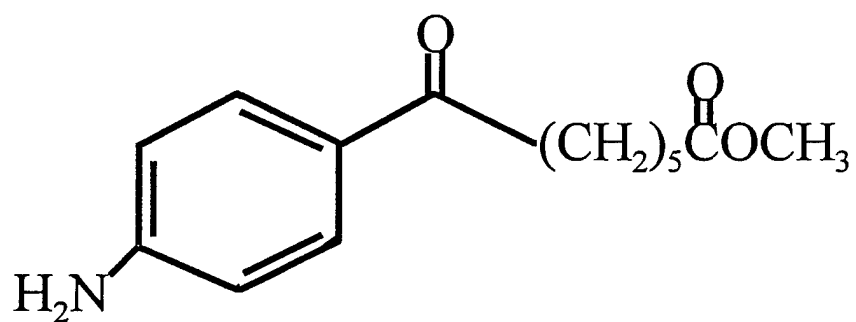Figure 12.   UV absorbance spectra of the PAHP urinary metabolite M1 and authentic PAHP.
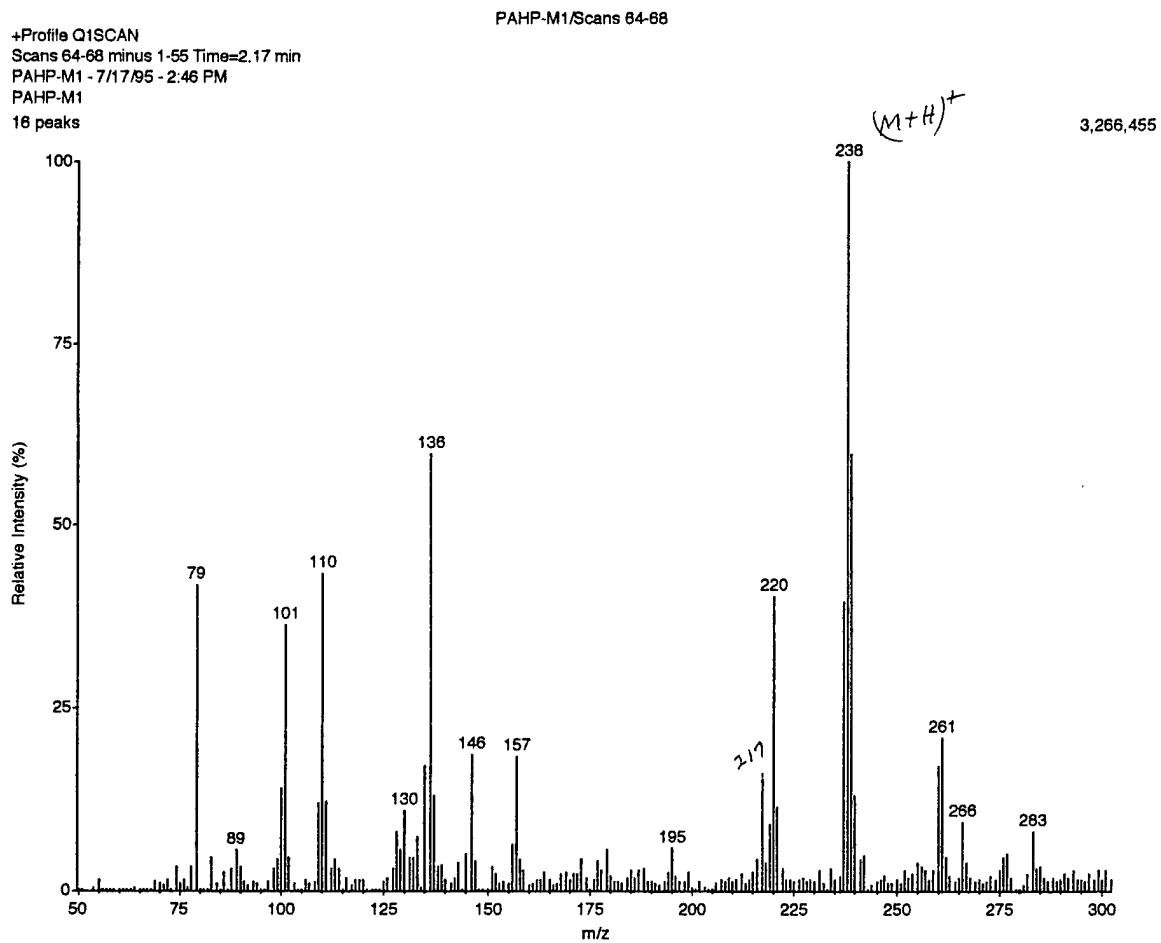
Figure 13.   Chemical ionization mass spectrum of the PAHP urinary metabolite M1.
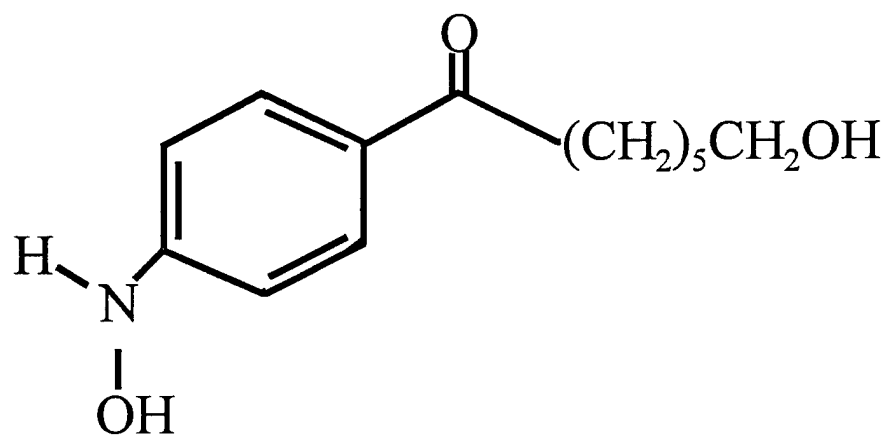
M1 (MW 237)
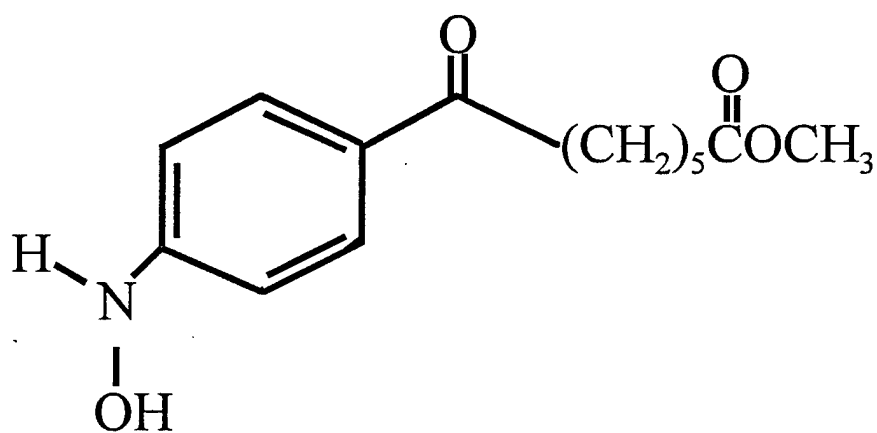
M2 (MW 265)

Figure 14.   Proposed structure of PAHP urinary metabolites M1 and M2.
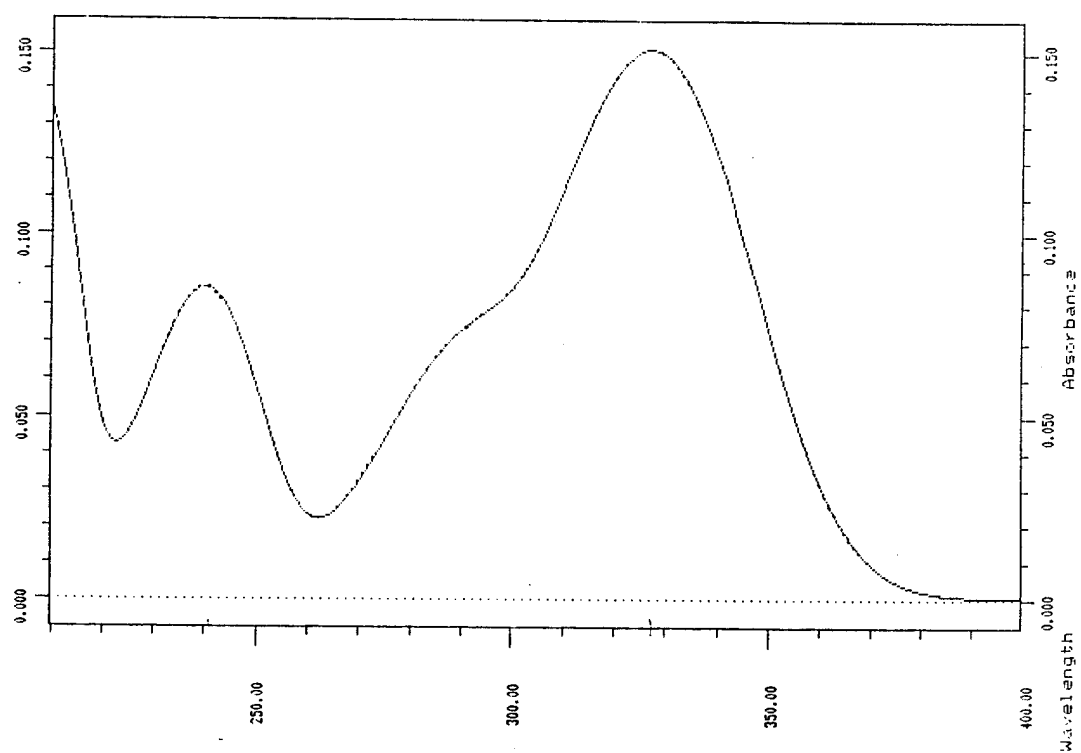
Figure 15.   UV absorbance spectrum of PAHP urinary metabolite M2.
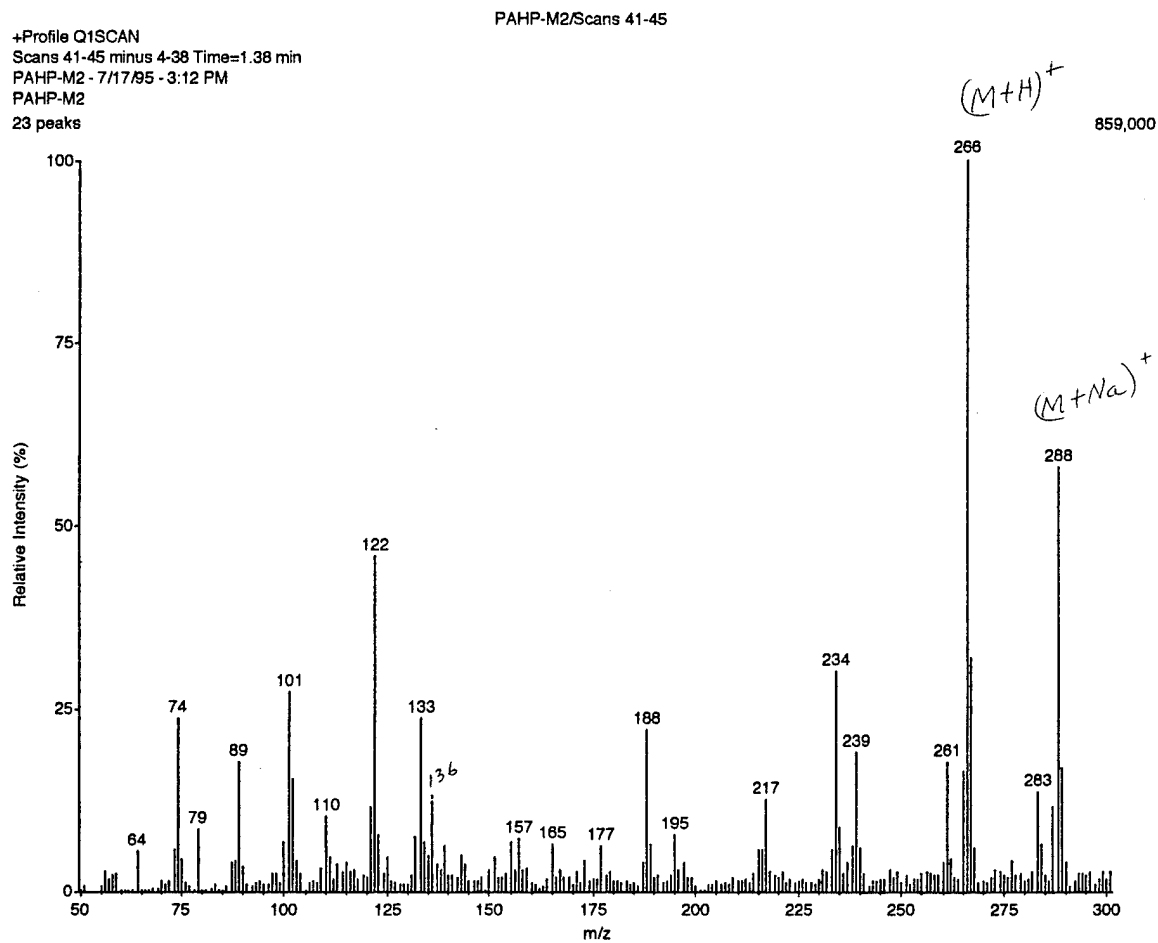
Figure 16.   Chemical ionization mass spectrum of the PAHP urinary metabolite M2.
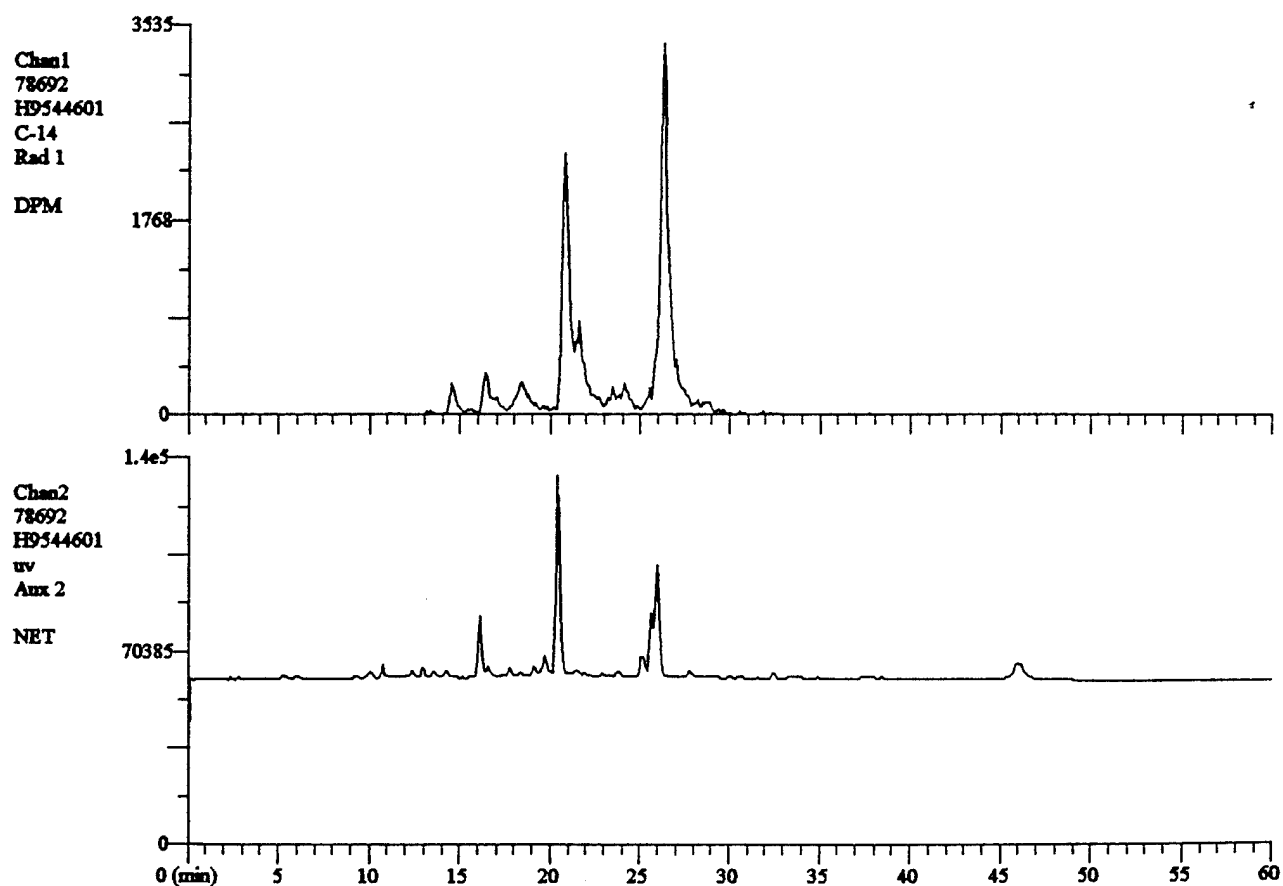
Figure 17.   Representative chromatogram obtained during the HPLC analysis of urine collected from a rat administered an i.v. dose of 7 mg/kg of [^{14}C]PAHP. The upper panel shows the radioactivity profile of the sample; the lower panel shows UV absorbance at 316 nm. In this system, PAHP eluted at 48 min.
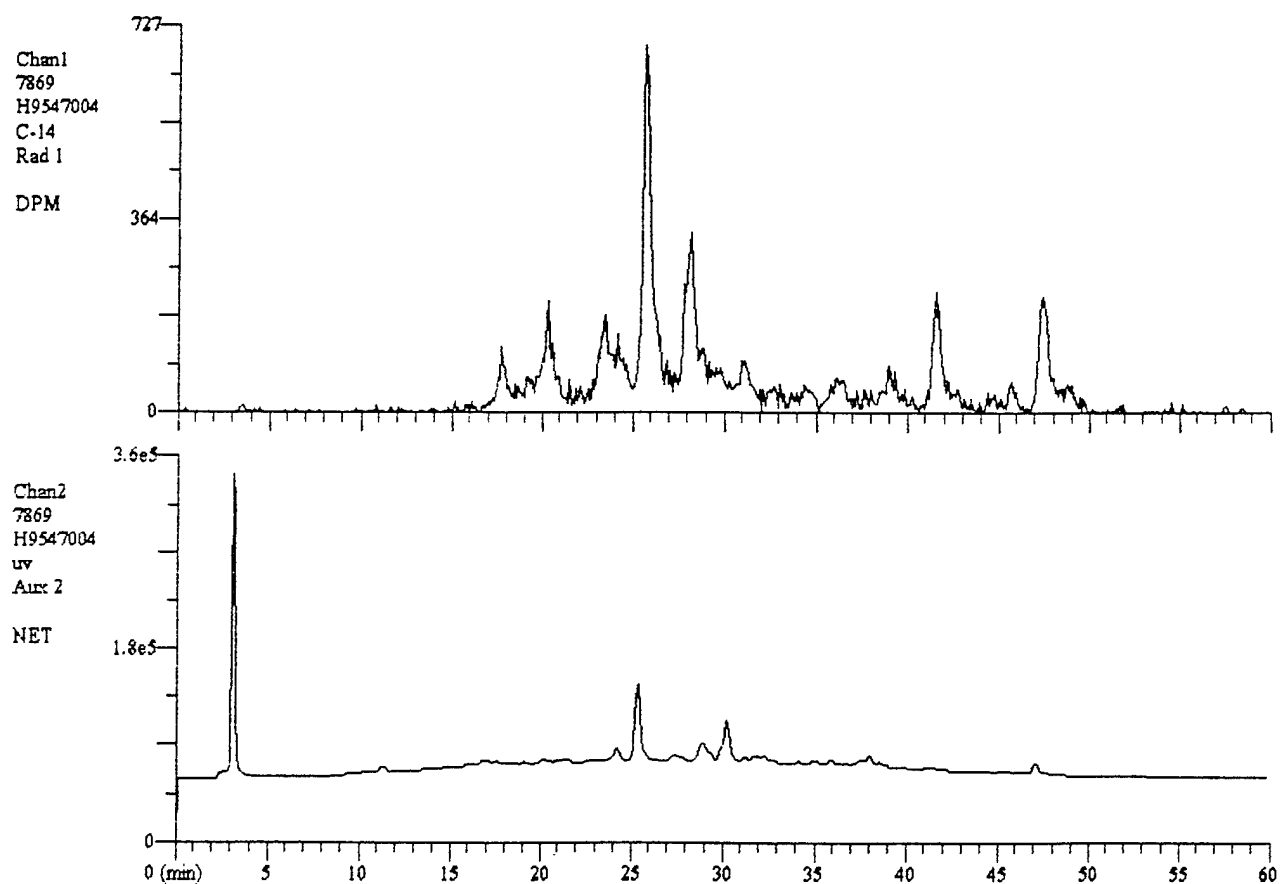
Figure 18.   UV absorbance spectra of the PAHP urinary metabolites M5 and M6.

1,218,069

250 (M+H)⁺

MW = 249

Relative Intensity (%)

100

75

50

25

0

102

232

133   204

267   (M+NH₄)⁺
234   (M+Na)⁺
272
283

516

100   200   300   400   500   600   700   800

m/z

Figure 19.   Chemical ionization mass spectrum of the PAHP urinary metabolite M6.

M6 (MW 249)

Figure 20.   Proposed structure of PAHP urinary metabolite M6.
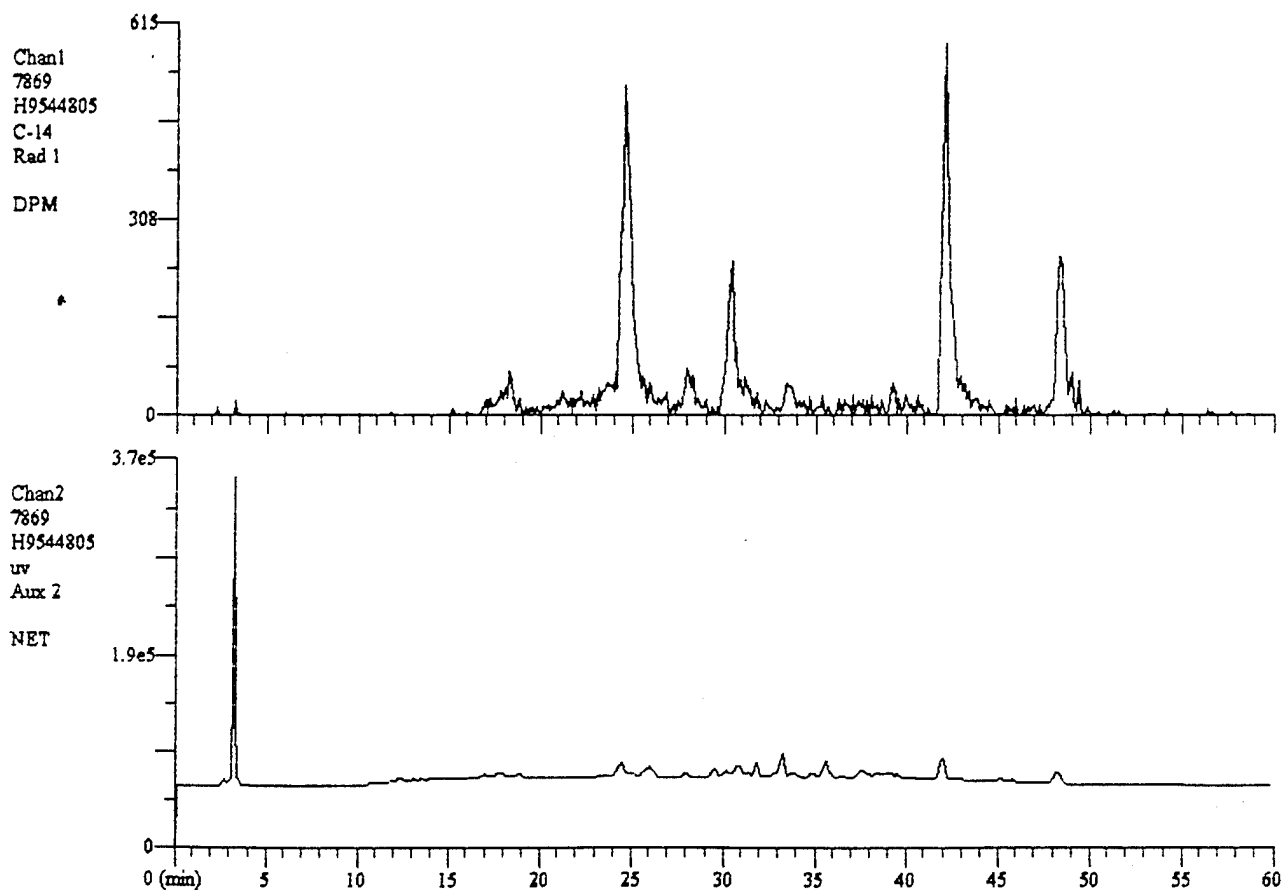
Figure 21. Representative chromatogram obtained during the HPLC analysis of feces collected
from a dog administered an i.v. dose of 7 mg/kg of [^{14}C]PAHP. The upper panel
shows the radioactivity profile of the sample; the lower panel shows UV
absorbance at 316 nm. In this system, PAHP eluted at 48 min.

Chan1
7869
H9547004
C-14
Rad 1

DPM

727
364
0

Chan2
7869
H9547004
uv
Aux 2

NET

3.6e5
1.8e5
0

0 (min)   5   10   15   20   25   30   35   40   45   50   55   60

Figure 22.   Representative chromatogram obtained during the HPLC analysis of feces collected from a rat administered an i.v. dose of 7 mg/kg of [^{14}C]PAHP. The upper panel shows the radioactivity profile of the sample;   the lower panel shows UV absorbance at 316 nm. In this system, PAHP eluted at 48 min.
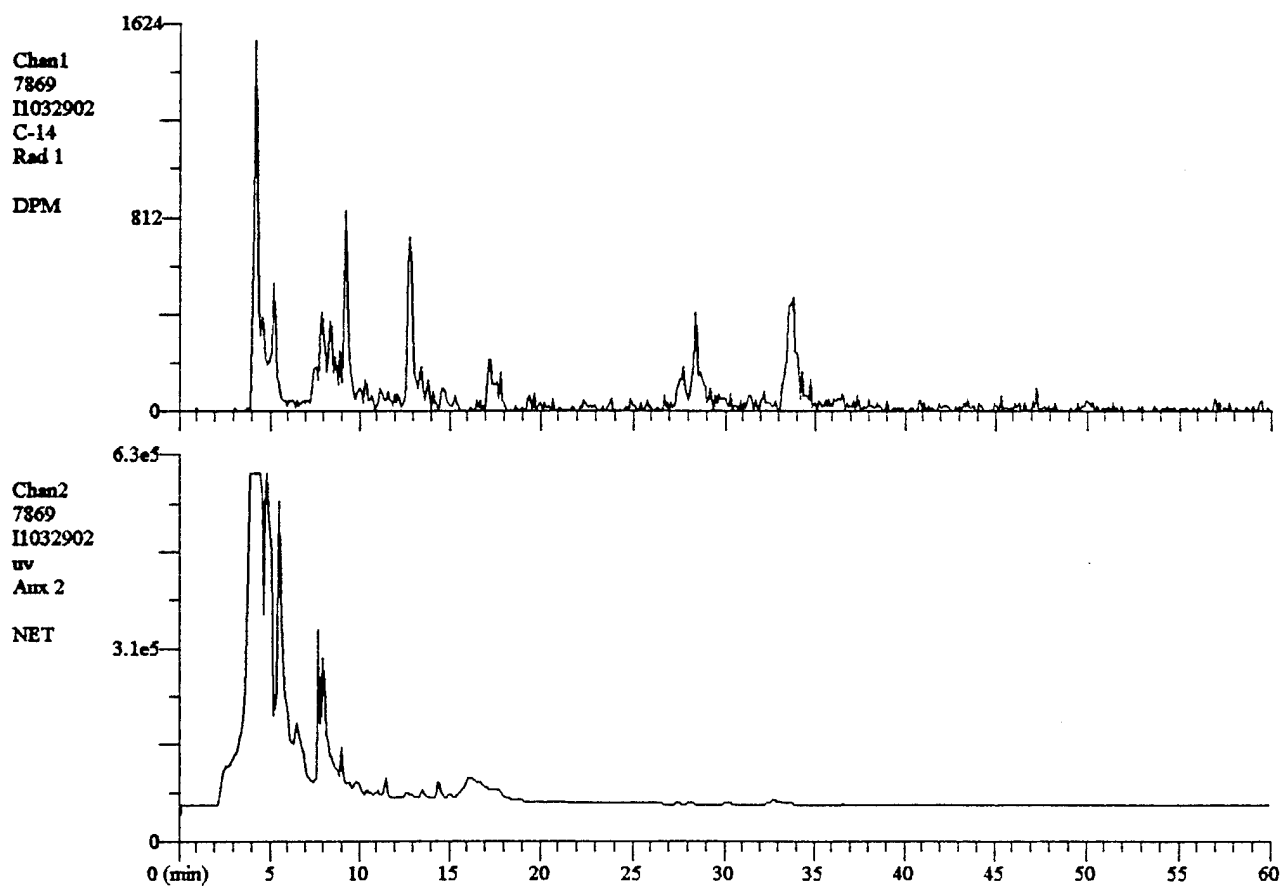
Figure 23. Representative chromatogram obtained during the HPLC analysis of urine collected from a dog administered 7 mg/kg of $[^{14}C]$WR242511. The upper panel shows the radioactivity profile of the sample; the lower panel shows UV absorbance at 262 nm.
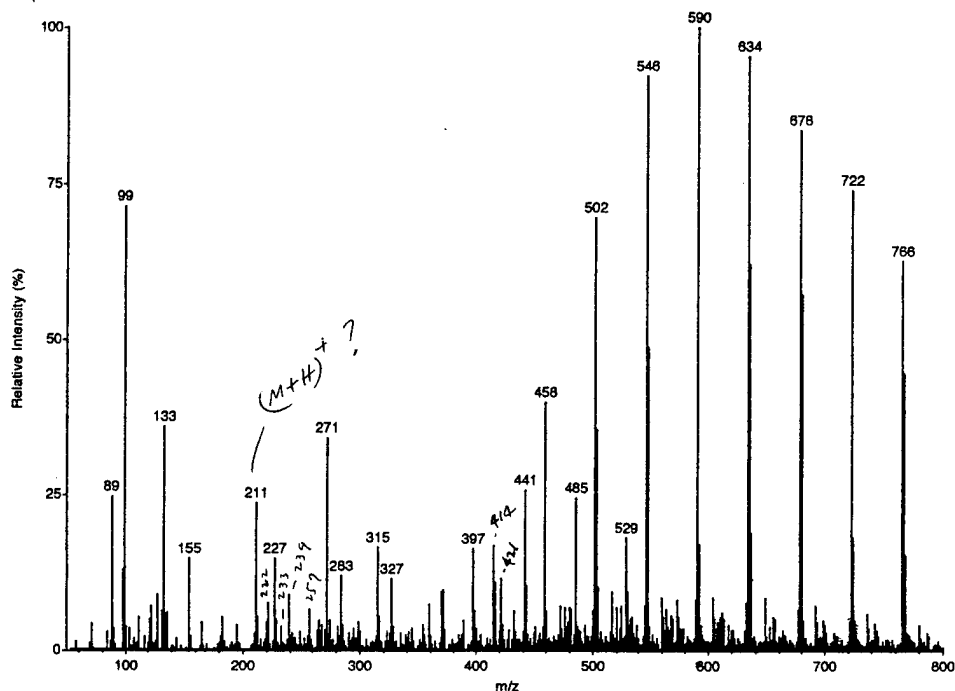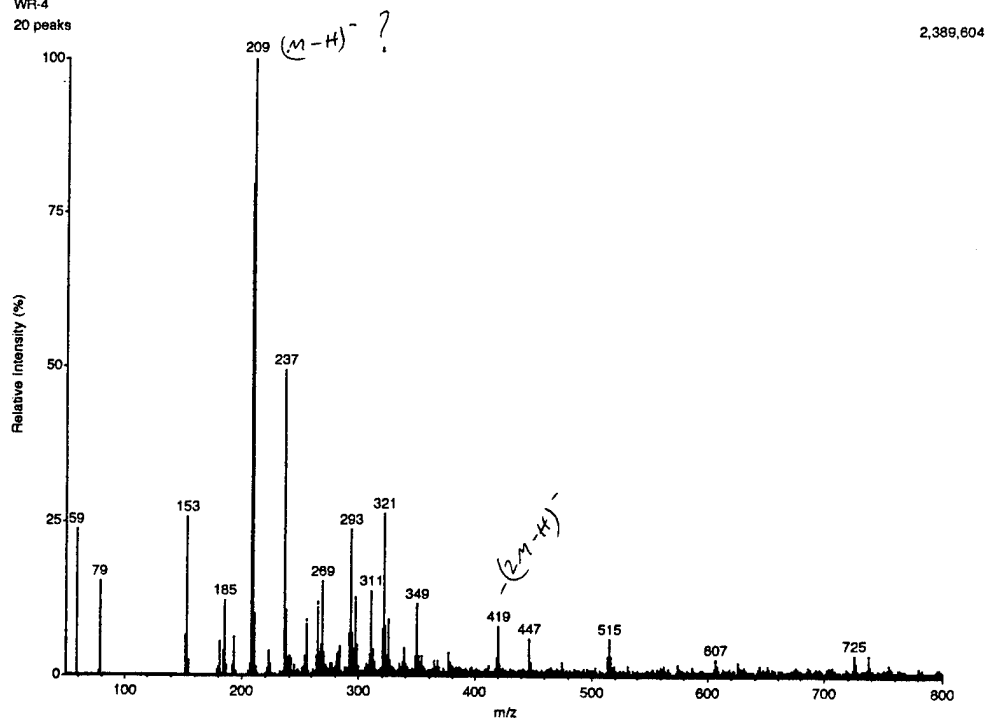
Figure 24.   Chemical ionization mass spectra of WR242511 metabolite WR4. The upper panel shows the positive mode spectrum; the lower panel, the negative mode spectrum.